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CONTENTS

Observations on the Possible Relation of Malignant Esophageal Tumors and *Spirocerca Lupi* Lesions in the Dog 5
H. R. Seibold, W. S. Bailey, B. F. Hoerlein, E. M. Jordan, and C. W. Schwabe

In Vitro Studies on the Anthelmintic Effect of Some Antimalarial Drugs 15
A. Sharaf and I. M. Shihata

Continuous Phenothiazine Therapy for Horses. II. A Toxonomic Study Following Four Years of Treatment 18
J. H. Drudge, Z. N. Wyant, and G. W. Elam

Antibody Response of Horses Following Intravenous Injection of Small Amounts of Active Japanese B Encephalitis Virus After Recent Natural Infection 22
K. F. Burns

The Pathogenesis of Telangiectasis in the Bovine Liver. I. The Incidence of Telangiectasis 27
A. C. Anderson

A Comparison of Regular Intradermic Johnin and a Purified Protein Derivative of Intradermic Johnin on Artificially and Naturally Sensitized Ruminants 35
A. B. Larsen, L. A. Baisden, and R. S. Merkal

The Teeth of the Ox in Clinical Diagnosis. IV. Dental Fluorosis 38
N. L. Garlick

Histopathological Findings in Cows Naturally Infected with *Leptospira Pomona* 48
W. J. Hadlow and H. G. Stoenner

The Effects of Some Avian Neurotropic Viruses on Young Dairy Calves 57
C. F. Helmboldt, R. E. Luginbuhl, A. H. Hammar, S. F. Satriano, and E. L. Jungherr

Plasma and Milk Tocopherol Levels of Cows Compared with the Plasma Tocopherol Levels of Their Foster Calves 64
J. W. Safford and K. F. Swingle

Changes in Temperature of Reticulo-Ruminal Content Following the Drinking of Water 69
R. D. Dillon and R. E. Nichols

An Investigation of Antibody Response in Cattle Vaccinated with the Rabbit-Passaged LA Rinderpest Virus in Korea 71
J. Nakamura, S. Kishi, J. Kiuchi, and R. Reisinger

A Preliminary Report on the Identification of *Vibrio Fetus* 76
J. H. Bryner and A. H. Frank

Physiological Effects of Insufflation of the Stomach of Sheep 79
R. W. Dougherty, C. D. Meredith, and R. B. Barrett

(Continued on page 3)

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Member-at-Large

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(Contents continued from cover)

The Effect of Age and Pregnancy on Ovine Blood Protein Fractions 91
J. S. Dunlap and W. M. Dickson

Cinefluorographic Studies of the Ruminant Stomach and of Eructation 96
R. W. Dougherty and C. D. Meredith

Further Research on the Prevention of Kidney Stones in Sheep from the Colloidal Approach 101
G. O. Puntriano

Observations on the Life Cycle of *Nematodirus Spathiger*, a Nematode Parasitic in the Intestiae of Sheep and Other Ruminants 105
K. C. Kates and J. H. Turner

The Effect of Transmissible Gastroenteritis on the Metabolism of Baby Pigs 116
E. F. Reber and C. K. Whitehair

Fragility of Erythrocytes in Blood from Swine of Two Age Groups 120
A. E. A. Hudson

Procurement of Baby Pigs by Hysterectomy 123
G. A. Young, N. R. Underdahl, and R. W. Hinz

Salmonellosis in Poultry and Poultry Processing Plants in Florida 132
M. M. Galton, D. C. Mackel, A. L. Lewis, W. C. Haire, and A. V. Hardy

The Use of Aqueous Humor for Early Diagnosis of Newcastle Disease 138
D. S. Clark, E. E. Jones, and F. K. Ross

Local Inhibition by Estrone of Testosterone-Induced Growth of the Capon's Comb 141
J. E. Martin, J. H. Graves, and F. C. Dohan

Electron Microscopic Studies of Laryngotracheitis Virus 147
R. L. Reagan, E. C. Delaha, S. R. Cook, A. L. Brueckner, J. E. Porter, and I. M. Moulthrop

Synthesis of Amino Acids from Urea by the Genus Brucella 149
H. S. Cameron and M. E. Meyer

A Comparative Study of the Structure of the Small Intestines of Domestic Animals 152
C. W. Titkemeyer and M. L. Calhoun

The Sterilizing Action of Gaseous Ethylene Oxide on Foot-and-Mouth Disease Virus—A Preliminary Report 158
M. Savan

Chemotherapeutic Properties of Erythromycin Alone and in Combination with Sulfamerazine 160
H. J. Florestano and M. E. Bahler

Serological and Physiological Relationships Between Strains of *Vibrio Fetus* 164
K. E. Price, L. J. Poelma, and J. E. Faber, Jr.

The Influence of Age of Human Tubercle Bacilli Cultures on the Potency of the Culture Fluids 170
L. A. Baisden, A. B. Larsen, and T. H. Vardaman

Polysaccharide Antigens from Three Leptospiral Serotypes 173
M. D. Schneider

Infectivity and Pathogenicity of Vesicular Stomatitis Virus for Ferrets 180
T. Kowalczyk, R. P. Hanson, and C. A. Brandly

Experimental Production of Hyperkeratosis in Rats and Hamsters 183
C. E. Schoettle, E. F. Reber, C. C. Morrill, and R. P. Link

Experimental *Vibrio Fetus* Infection in Male Hamsters 189
M. Ristic, D. A. Sanders, and F. Young

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VOL. XVI JANUARY, 1955 No. 58

Observations on the Possible Relation of Malignant Esophageal Tumors and *Spirocerca Lupi* Lesions in the Dog

H. R. SEIBOLD, V.M.D.; W. S. BAILEY, D.V.M., M.S., Sc.D.;
B. F. HOERLEIN, D.V.M., Ph.D.; E. M. JORDAN, D.V.M.;
C. W. SCHWABE, M.S., D.V.M.

Auburn, Alabama

ACCORDING to Mulligan[1] tumors of the esophagus in the dog are rare. Leiomyoma and squamous cell carcinoma have been described. Jackson[2] mentioned one fibrosarcoma in the esophagus of the dog. Feldman[3] did not specifically mention any canine esophageal tumor.

The purpose of this report is to describe a series of seven malignant connective tissue tumors in the esophagus of dogs and to present evidence indicating the origin of these neoplasms in lesions produced by *Spirocerca lupi*, the esophageal worm. Two of the tumors were fibrosarcomas, and five were osteosarcomas. Three of the osteosarcomas had metastasized. All of the tumors originated in the posterior portion of the esophagus in the region where Spirocerca usually locate in dogs. In 4 cases (1 fibrosarcoma and 3 osteosarcomas) there were nodules containing Spirocerca either in the esophagus or in the aorta and in close approximation with the tumor. The 4 dogs in which both tumor and Spirocerca lesions were found were the 4 cases last observed. In retrospect, it seems probable that Spirocerca lesions were overlooked in dogs 1 and 3 of this tumor series. That assumption is based on the fact that these dogs, like the last 4, showed deformative ossifying spondylitis (spondylitis deformans ossificans) of the bodies of several of the posterior thoracic vertebrae (no record on this point was made for dog 2, the only outside case). This lesion is undoubtedly the result of an irritation of the periosteum caused by the migration of the parasite. The essential pathological observations are summarized in table 1.

Another point of interest was the presence of hypertrophic pulmonary osteoarthropathy in the 2 dogs with fibrosarcoma and in 1 with osteosarcoma.

DESCRIPTION OF CASES

Dog 1.—An 8-year-old male setter was presented at the Small Animal Clinic on June 15, 1951. The dog would eat and then vomit, had become sluggish, urinated frequently but did not drink excessively. He had greatly enlarged legs and feet. Radiographs below the elbow and stifle joints showed periosteal exostosis characteristic of hypertrophic pulmonary osteoarthropathy. Chest radiographs revealed a large tumor-like mass of the esophagus situated directly below lesions of deformative ossifying spondylitis on the sixth and seventh thoracic vertebrae. Clinical laboratory examination revealed (*a*) microfilaria; (*b*) blood urea nitrogen (b.u.n.): 10 mg. per 100 cc.; (*c*) red blood corpuscles (r.b.c.): 3,700,000 per cu. mm.; (*d*) white blood corpuscles (w.b.c.): 44,700 per cu. mm. (first examination), 59,450 per cu. mm. (second examination), neutrophils 94% (mature), lymphocytes 6%, many platelets.

The dog was observed for eight days before euthanasia was recommended.

Necropsy revealed a mushroom-like proliferation from the mucosa of the posterior mediastinal esophagus about 10 cm. in diameter and 3 cm. thick (fig. 1) composed of firm white tissue, its lumen surface ulcerated; adult heart worms in the

Approved by the Committee on Publications, School of Veterinary Medicine, Alabama Polytechnic Institute, publication No. 437. From the Department of Pathology and Parasitology (Seibold and Bailey), Department of Small Animal Surgery and Medicine (Hoerlein and Jordan), Department of Bacteriology (Schwabe). Dr. Schwabe's present address is Department of Tropical Public Health, Harvard University, Boston, Mass.

TABLE 1—Summary of Pathological Observations on Relation of Malignant Esophageal Tumors and Spirocerca Lupi Lesions in the Dog

Case (No.)	Breed	Sex	Age (yr.)	Type of tumor	Metastasis	*Spirocerca lupi*	Thoracic spondylitis	Osteoarthropathy
1	Setter	M	8	Fibrosarcoma	None	No record	Present	Present
2	Pointer	F	6	Osteosarcoma	No necropsy	No record	No record	Not present
3	Hound	F	9	Osteosarcoma	Lungs, bronchial nodes, pleura, kidneys	Probable *Spirocerca* lesions	Present	Not present
4	Hound	M	10	Fibrosarcoma	None	Present	Present	Present
5	Hound	M	7	Osteosarcoma	Lungs, bronchial nodes, anterior mediastinum	Present	Present	Not present
6	Hound	M	3½	Osteosarcoma	None	Present	Present	Not present
7	Hound	M	8	Osteosarcoma	Lungs, bronchial nodes, pericardium, liver	Present	Present	Present

right ventricle; some pulmonary anthracosis; and moderate chronic interstitial nephritis. In addition to osteoarthropathy, there was a ventral bony protuberance of the bodies of the sixth and seventh thoracic vertebrae—a deformative ossifying spondylitis that was subsequently noticed in dogs infected with Spirocerca.

Histopathology.—Sections of the esophageal tumor had the appearance of a fibrosarcoma characterized by small, plump spindle cells arranged in whorls and bundles (fig. 2), a small amount of uniformly distributed intercellular collagenous stroma, and numerous mitotic figures. The ulcerated surface showed a narrow zone of necrosis and inflammatory cellular infiltration.

Diagnosis.—For dog 1 the diagnosis was fibrosarcoma of the esophagus, osteoarthropathy, heartworms, anemia, and localized thoracic deformative ossifying spondylitis.

Dog 2.—A 6-year-old female pointer was pre-

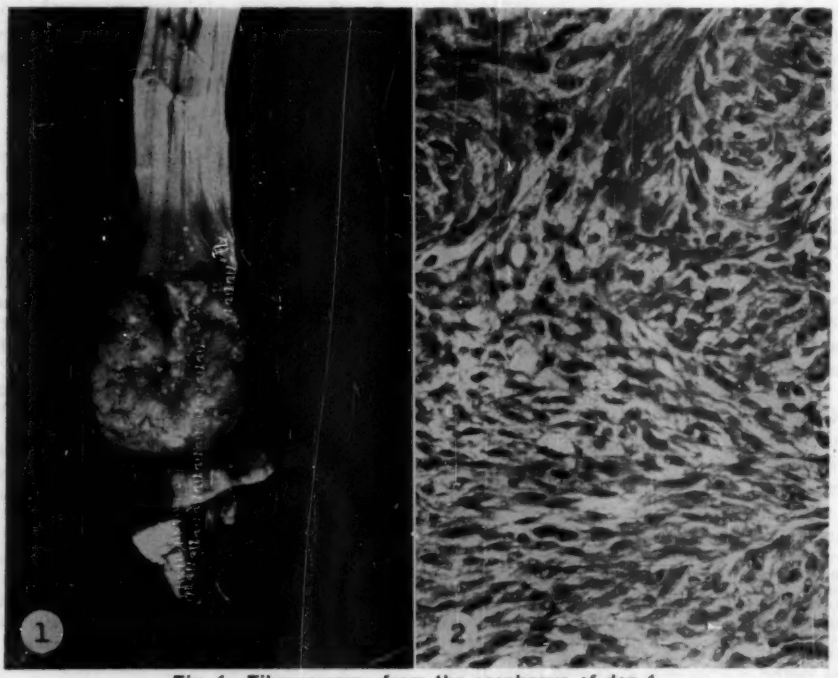

Fig. 1—Fibrosarcoma from the esophagus of dog 1.
Fig. 2—Typical area of fibrosarcoma from the esophagus of dog 1. x 290. (AFIP 545,385).

sented at a hospital in Anniston, Ala., on Aug. 19, 1952.*

The dog for six weeks had vomited immediately after eating, was underweight, dehydrated, and had abnormal chest sounds. Radiographs of the

Fig. 3—Chest radiograph of dog 3. The obstruction in the esophagus is revealed by the failure of the $BaSO_4$-filled capsule to pass. A marked deformative ossifying spondylitis is seen adjacent to the tumor.

chest revealed a mass that was either an esophageal tumor or a tumor of the diaphragmatic lobe of the lung pressing on the esophagus. No clinical laboratory data were available. When surgical exploration of the chest revealed a large tumor in the posterior portion of the esophagus, it was resected, and an end-to-end anastomosis was done. The dog died in shock on the following day, no necropsy being made.

Histopathology.—The tumor was composed of coalescing nodules, some soft and cellular, others partially or completely bony. The soft nodules consisted of interlacing bundles of medium-sized spindle cells and a mixture of polyhedral osteoblast-type cells and multinucleated osteoclast-type cells scattered in patches and linear fashion among the spindle cell bundles. A variable amount of osseous intercellular substance was deposited among the osteoblast-type cells. Numerous mitotic figures were present. A majority of the tumor nodules showed a greater degree of differentiation into atypical bone tissue than the soft nodules described above. The surface of the growth was ulcerated, and a considerable area of subjacent tumor tissue was necrotic and infiltrated with leukocytes. Without a necropsy, metastasis in this case was not determined.

Diagnosis.—For dog 2 the diagnosis was osteosarcoma of the esophagus.

* The authors are indebted to Dr. W. N. Konde for the specimen of esophageal tumor and the data pertaining to this case.

Dog 3.—A 9-year-old female hound presented on April 3, 1953, had lost weight during the previous two months, during which time it "strangled" when swallowing. Radiographs revealed neoplasms in the esophagus, lungs, and kidneys, and deformative ossifying spondylitis adjacent to the esophageal tumor (fig. 3). Clinical laboratory examination revealed (*a*) r.b.c.: 4,940,000 per cu. mm.; (*b*) w.b.c.: 13,450 per cu. mm., neutrophils, 52%, lymphocytes 48%; (*c*) packed r.b.c. volume: 4.2 mm.; (*d*) hemoglobin (Hb.): 15 Gm.; (*e*) sedimentation rate: 0.5 mm./30 min., 1.6 mm./1 hr.; (*f*) b.u.n.: 7.7 mg. per 100 cc.

No treatment was attempted, and euthanasia was performed.

Necropsy revealed a large, apparently primary, tumor 5 cm. in diameter projecting into the lumen in the posterior part of the esophagus (fig. 4). On section, this was firm and greyish white at the periphery and cut as though it contained bone spicules. The portion projecting into the lumen was ulcerated, with the cavity of the ulcer extending into the necrotic center of the growth. Small metastatic bony tumor nodules were in all lobes of the lungs and in the bronchial lymph nodes and kidneys; similar but smaller nodules were on the pleural surface of the epicardium and on the parietal pleura.

Spirocerca infection was not recorded, but pictures of the tumor show two small nodular elevations typical of Spirocerca lesions in the esopha-

Fig. 4—Osteosarcoma in esophagus of dog 3. Notice two nodules (probably due to Spirocerca lupi) immediately below tumor.

geal mucosa in proximity to the growth (fig. 4).

Histopathology.—The growth in the esophagus was composed of coalescent nodules of neoplastic tissue. A few nodules were highly cellular, consisting of interlacing bundles of medium-sized spindle cells with a small amount of collagenous intercellular substance (fig. 5) but also showing small areas where chondroid or osteoid intercellular substances were developing. A majority of the neoplastic nodules consisted of a central area of bony trabeculae and a peripheral area of sarcomatous tissue composed of medium-sized spindle and polyhedral cells. Numerous mitotic figures were seen in the peripheral areas, where growth apparently was more active. The bony trabeculae in the center were bordered by polyhedral cells resembling osteoblasts; there also were individual osteoclast-type cells scattered about (fig. 6). Metastatic tumors in the lung, kidney, and bronchial lymph node resembled closely the more osseous type nodules in the primary esophageal growth.

Diagnosis.—For dog 3 the diagnosis was osteosarcoma of the esophagus with metastasis to the lungs, pulmonary lymph nodes, pleura, and kidneys and deformative ossifying spondylitis adjacent to the tumor.

Dog 4.—A 10-year old male hound was presented on May 11, 1953, with feet and legs swollen and skin lesions described by the owner as a "breaking out over the body." Radiographs revealed lesions of osteoarthropathy on the bones of the extremities (fig. 7), deformative ossifying spondylitis affecting the bodies of the fifth, sixth, seventh, and eighth thoracic vertebrae, and a large opaque mass below the affected vertebrae that was suspected of being a tumor. Fecal examination revealed a moderately heavy hookworm infection. Clinical laboratory examination revealed (*a*) b.u.n.: 5.9 mg. per 100 cc.; (*b*) serum calcium: 8.5 mg. per 100 cc.; (*c*) serum phosphorus: 4.6 mg. per 100 cc.; (*d*) r.b.c.: 3,960,000 per cu. mm.; (*e*) w.b.c.: 17,400 per cu. mm., neutrophils 78%, lymphocytes 20%, eosinophils 2%; (*f*) Hb.: 12 Gm.

The dog was treated with penicillin for a month, then euthanasia was performed.

Necropsy revealed a carcass in good physical condition with enlargement of all the bones distal to the humerus and femur characteristic of osteoarthropathy; a polyp 3 x 2 x 2 cm. in the esophagus about 8 cm. anterior to the stomach; a nodule containing Spirocerca in the wall of the esophagus near the base of the polyp, and directly at the base of the polyp a fibrous nodule which was judged

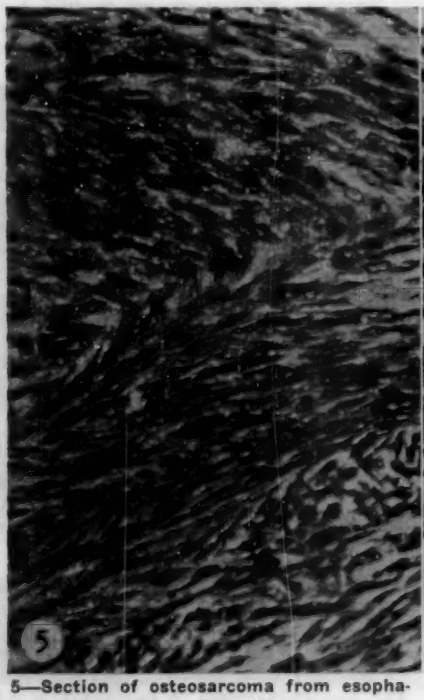

Fig. 5—Section of osteosarcoma from esophagus of dog 3 showing cellular area in primary tumor. x 290. (AFIP 545,384).

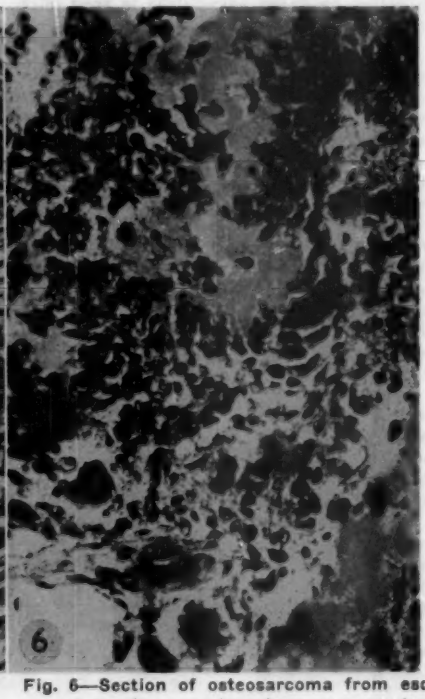

Fig. 6—Section of osteosarcoma from esophagus of dog 3; areas in primary tumor showing bone development. x 290. (AFIP 545,381).

to be an old Spirocerca lesion. The prostate was somewhat enlarged.

Histopathology.—The polyp in the esophagus was composed of large, anaplastic, malignant fibroblastic cells arranged in whorls and bundles. Numerous mitotic figures were seen. Sections of the enlarged bones of the extremities revealed irregular thickening of the cortex by proliferation of exostosis-like accumulations of bone and cartilage. There was passive congestion of the liver, and the lung showed patches of bronchopneumonia.

Diagnosis.—For dog 4 the diagnosis was fibrosarcoma of the esophagus, Spirocerca infection, deformative ossifying spondylitis, and osteoarthropathy.

Dog 5.—A 7-year-old male hound, presented on Nov. 30, 1953, had been vomiting all food for more than a week, appeared emaciated, and was salivating profusely when first examined. When hand feeding was attempted, regurgitation occurred approximately 15 seconds after swallowing. Labored breathing developed when the dog was exercised. Radiographs of the thorax revealed two large tumors (fig. 8) and, after the administration of a barium meal, demonstrated that one of the tumors was definitely in the esophagus. Results of the clinical laboratory examination were (*a*) r.b.c.: 3,819,000 per cu. mm.; (*b*) w.b.c.: 24,900 per cu. mm., neutrophils 94%, lymphocytes 5%, eosinophils 1%; (*c*) b.u.n.: 9.54 mg. per 100 cc.; (*d*) urinalysis: specific gravity 1.061, sugar: +, albumin: +, indican: +++; also (microscopic) w.b.c.'s, yeast, bladder and renal epithelial cells.

Euthanasia was performed on December 2.

Necropsy revealed a carcass in poor condition; the lungs containing numerous metastatic bony tumor nodules 2 to 5 mm. in diameter; one bronchial lymph node containing a bony tumor nodule 1½ cm. in diameter; and several confluent tumors about 3 cm. in total diameter in the anterior mediastinum. These apparently correspond to one of the tumors seen in the radiograph. The primary tumor, located in the dorsal wall of the posterior portion of the esophagus, was about 7 cm. in diameter with a necrotic center which communicated with the lumen of the esophagus through a large ulcer. The posterior aorta had several small nodular elevations on the adventitial surface, some of which contained Spirocerca. The posterior aorta also showed considerable aneurysmal scarring characteristic of the damage caused by the migration of Spirocerca. The bodies of several adjacent thoracic vertebrae showed the deformative ossifying spondylitis associated with Spirocerca infection.

Histopathology.—The primary tumor in the wall of the esophagus was an osteosarcoma composed of stellate and polyhedral malignant osteoblasts and a variable amount of intercellular osseous stroma (fig. 9). The tumor lobules were much more cellular at the periphery than in the center, where ossification was taking place. Numerous mitotic figures were seen. The primary growth appeared to have originated in an area of scar tissue permeated with pus pockets, which was probably an old Spirocerca lesion. The metastatic tumor nodules had the same general histological appearance as the primary osteosarcoma (fig. 10).

The wall of the aorta contained areas of scar tissue, and the adventitia in one place contained two fibrous nodules enclosing Spirocerca. There was passive congestion of the liver. The kidneys showed a few microfilaria in the glomerular capillaries.

Diagnosis.—For dog 5 the diagnosis was osteosarcoma of the esophagus with metastasis, Spirocerca infection with associated aneurysmal scarring of the posterior aorta, and regional deformative ossifying spondylitis.

Dog 6.—A 3½-year-old male hound was brought to the clinic on Jan. 6, 1954, because of chronic vomition. Radiographs revealed an opacity about 8 cm. in diameter in the same location where tumors were found in the previous cases (fig. 11). Deformative ossifying spondylitis was evident above the tumor on the seventh, eighth, ninth, and tenth thoracic vertebrae with slight lesions on the elev-

Fig. 7—A radiograph showing subperiosteal exostosis from dog 4 characteristic of hypertrophic pulmonary osteoarthropathy.

Fig. 8 — Esophageal tumor from dog 5 in a normal contrast radiograph. The relations of the organs are the same as those sketched in figure 11.

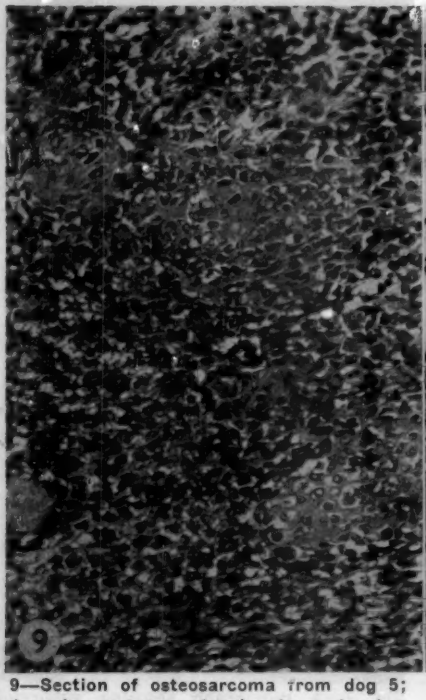

Fig. 9—Section of osteosarcoma from dog 5; area in primary tumor showing bone development. x 150. (AFIP 545,388).

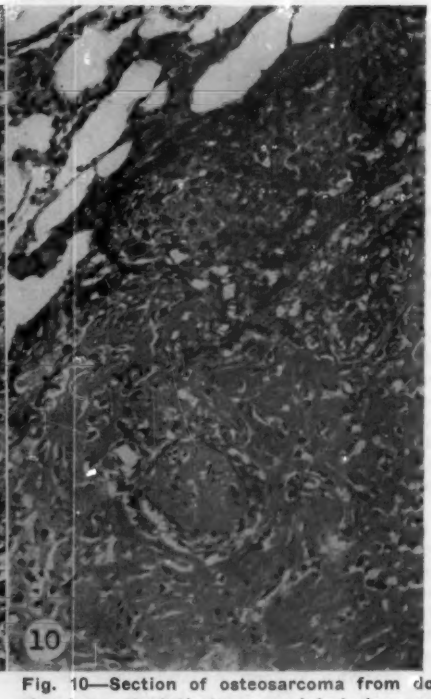

Fig. 10—Section of osteosarcoma from dog 5 showing metastatic tumor nodule in lung. x 150. (AFIP 545,390).

enth and twelfth thoracic vertebrae. Part of a barium meal was retained for some time in the esophageal lesion. No clinical laboratory tests were made. A tentative diagnosis of esophageal tumor was confirmed by intrathoracic surgery, a tumor 7½ cm. in diameter being removed from the posterior portion of the esophagus. The dog succumbed about thirty-six hours after the operation.

Necropsy revealed no indications of metastasis, and one Spirocerca lesion in the wall of the esophagus posterior to the tumor and near the entrance into the stomach (fig. 12). The intimal surface of the aorta, in the posterior thoracic region, had two roughened areas about 0.5 cm. in diameter. The ventral portion of the bodies of several nearby thoracic vertebrae showed irregular exostoses or deformative ossifying spondylitis apparently the result of Spirocerca migration.

Fig. 11A—Chest radiograph of dog 6 showing the esophageal tumor before barium meal administration.

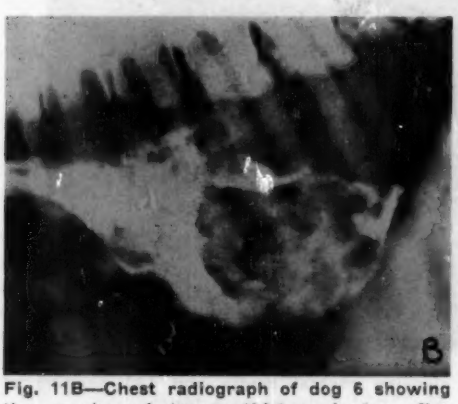

Fig. 11B—Chest radiograph of dog 6 showing the esophageal tumor thirty minutes after barium meal administration.

Histopathology.—The tumor was composed of spindle cells arranged in whorls and bundles and numerous areas of polyhedral cells that were producing either cartilaginous or osseous-type intercellular stroma. There were many mitotic figures, and the growth appeared to be quite invasive. No metastases were seen in the sections of the lung. The aortic lesion was a scar, presumably from Spirocerca migration.

Diagnosis.—For dog 6 the diagnosis was osteosarcoma of the esophagus, Spirocerca infection, and deformative ossifying spondylitis.

Dog 7.—An 8-year-old male hound, obtained from the vicinity of Lafayette, Ala.,* was reported to have been sick only about two weeks yet, when examined, all four limbs showed the characteristic enlargement of hypertrophic pulmonary osteoarthropathy, the subperiosteal bone deposits showing well in radiographs. Radiographs of the thorax revealed a large mass in the posterior esophageal region and exostosis or deformative ossifying spondylitis of the ventral aspect of the sixth and seventh thoracic vertebrae directly above the esophageal mass. The relationship between Spirocerca infection and esophageal tumors having now been recognized, the feces were examined and Spirocerca eggs and many hookworm eggs were found. Clinical laboratory examination revealed (*a*) r.b.c.: 1,860,000 per cu. mm.; (*b*) w.b.c.: 23,500 per cu. mm., neutrophils 79%, lymphocytes 7%, monocytes 1%, eosinophils 2%; (*c*) packed r.b.c. volume: 15%; (*d*) Hb.: 7 Gm.; (*e*) sedimentation rate: 20 mm./30 min.; (*f*) b.u.n.: 10.5 mg. per 100 cc.

Euthanasia was performed the following day.

Necropsy revealed a carcass in poor condition, marked osteoarthropathy of all four limbs, and an apparently primary tumor 5½ x 4 x 3½ cm. in the

* The authors are indebted to Dr. John Durr for the opportunity to study this case.

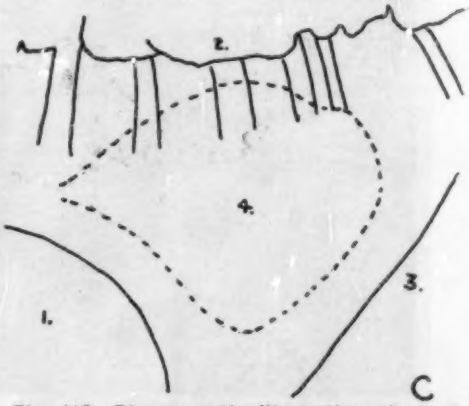

Fig. 11C—Diagrammatic illustration of structures seen in radiographs. (1) heart; (2) deformative ossifying spondylitis; (3) diaphragm; (4) esophageal tumor.

dorsal wall of the esophagus about 1 cm. anterior to the diaphragm with a cavity about 4½ cm. long and 1½ cm. deep in communication with the lumen of the esophagus through an ulcer (fig. 13). The lining of the cavity was necrotic, and it was filled with decayed food and necrotic debris. In the esophageal mucosa adjacent to the ulcer was a 3-mm. greyish nodule from which, through a small opening, fluid containing Spirocerca eggs was expressed (A in fig. 13). Transverse section of this nodule revealed a small cavity in the edge of the tumor filled with several adult Spirocerca (fig. 14). Other findings included hard, white, metastatic tumor nodules 2 to 8 mm. in diameter in all lobes of the lungs (fig. 15), the bronchial lymph nodes, the pericardium, and the liver; grape-like clusters of small hard metastatic tumor nodules on the anterior and posterior mediastinum; and aneurysmal scarring from Spirocerca migration in the aorta above the primary esophageal tumor. Regional deformative ossifying spondylitis was seen on radiographs.

Histopathology.—The primary tumor was composed of neoplastic bony tissue consisting of large anaplastic osteoblast-type cells and an irregular network of intercellular bony matrix. The tumor tissue formed a thick shell around the cavity and was covered by a markedly thickened esophageal tunica adventitia. One lobule of tumor tissue was in continuity with the tissue around a Spirocerca cyst. Metastatic tumor nodules in the lungs, liver, bronchial lymph nodes, pericardium, and mediastinal pleura were practically identical with the primary growth except for being somewhat more cellular and containing a larger number of mitotic figures. The wall of the aorta showed a marked but irregularly distributed increase of fibrous tissue.

Diagnosis.—For dog 7 the diagnosis was osteosarcoma of the esophagus with metastasis, Spirocerca infection, deformative ossifying spondylitis, and osteoarthropathy.

DISCUSSION

The apparent relation of Spirocerca lesions to the genesis of malignant esophageal tumors was not recognized until after the fourth case in this series had been studied. The first case, a fibrosarcoma with osteoarthropathy, was considered of interest only because the tumor related to

Fig. 12—Incised Spirocerca lesion in the wall of the esophagus of dog 6 between site of tumor (indicated by surgical incisions) and gastric mucosa.

Fig. 13—Osteosarcoma in esophagus of dog 7 showing central cavity and (A) opening of Spirocerca lesion.

osteoarthropathy was located in the esophagus. In the second and third cases, osteosarcomas without osteoarthropathy, it was considered remarkable that osteosarcoma

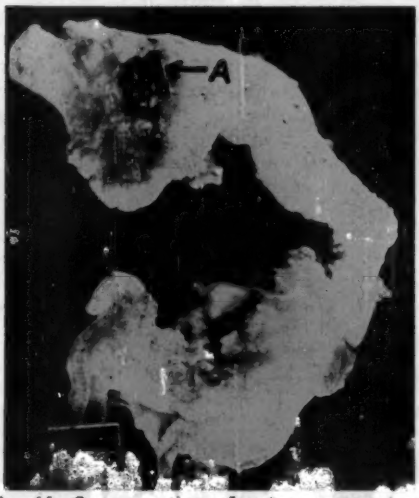

Fig. 14—Cross section of osteosarcoma from dog 7 showing incised Spirocerca lesion (A) containing adult worms.

should develop in the esophagus. The first diagnosis of osteosarcoma was made with considerable reservation; the second diagnosis was well established by the metastatic tumors in the lungs and elsewhere.† However, in retrospect the presence of deformative ossifying spondylitis in the thoracic region of dogs 1 and 3 assumes significance, especially since there was no osteoarthropathy in dog 3. This localized deformative ossifying spondylitis was seen in all cases in this series entered through the Small Animal Clinic (6 out of 7). Spondylitis most frequently occurs in the lumbar and cervical regions, the areas where the spinal column is most flexible and therefore most subject to "wear and tear." Only in the most severe cases of widespread deformative ossifying spondylitis are the thoracic vertebrae ever affected. Therefore, it seems most significant that these cases should all show the spinal lesion as a localized manifestation directly above the esophageal tumor.

† All the tumors in this series have been reviewed by the Registry of Veterinary Pathology, Armed Forces Institutes of Pathology, Washington, D. C.

The first instance in which Spirocerca was noticed in relation to a malignant esophageal tumor was case 4, a fibrosarcoma with osteoarthropathy, which also showed deformative ossifying spondylitis above the region where the tumor and the parasitic lesion were located. This close association of a tumor, Spirocerca, and deformative ossifying spondylitis was then demonstrated in the 3 subsequent cases. It is significant that the deformative ossifying spondylitis occurring in relation to Spirocerca was present in all 4 instances in which the parasite was found, while only 2 of these cases had osteoarthropathy, indicating that the spondylitis was related to the Spirocerca rather than to the osteoarthropathy. It is also significant that all seven tumors occurred in the region of the esophagus where Spirocerca lesions characteristically occur. The significance of Spirocerca infection in the genesis of malignant esophageal tumors is illustrated (tables 2 and 3) in figures based on a to-

Fig. 15—Surface view of osteosarcoma from dog 7 showing metastatic tumor nodules in lung.

TABLE 2—Comparative Incidence of Esophageal Tumors in Dogs Infected with Spirocerca Lupi (Worms Demonstrated) and Noninfected Dogs

No. of dogs autopsied	943
No. of dogs infected with *S. lupi*	39
No. of dogs with esophageal tumor	6
Percentage of dogs with tumors	0.6
Percentage of *S. lupi*-infected dogs which have tumors	10.2
Percentage of *S. lupi*-negative dogs which have tumors	0.2

tal of 943 dogs autopsied during the period between case 1 and case 7 inclusive, and on a total of six esophageal tumors, the one submitted as a biopsy specimen being omitted. Table 2 compares the percentage of

TABLE 3—Comparative Incidence of Esophageal Tumors in Dogs Infected with Spirocerca Lupi (When Deformative Ossifying Spondylitis Is Considered Indication of Infection) and Noninfected Dogs

No. of dogs autopsied	943
No. of dogs considered to be infected with *S. lupi**	41
No. of dogs with esophageal tumors	6
Percentage of dogs with tumors	0.6
Percentage of *S. lupi*-infected dogs which have tumors	14.6
Percentage of *S. lupi*-negative dogs which have tumors	0.0

* 39 known infected plus 2 considered to be infected on basis of deformative ossifying spondylitis.

tumors in infected and noninfected dogs when only the 4 dogs in which the parasite was actually found are considered to be infected. Table 3 shows the comparative incidence in infected and noninfected groups when spondylitis deformans is considered indicative of Spirocerca infection.

Regardless of the interpretation of the deformative ossifying spondylitis, it appears that there is a highly significant difference in the percentage of esophageal tumors among Spirocerca infected and noninfected dogs. The data presented in this paper thus indicate that Spirocerca infection is an inciting factor of malignant esophageal tumors.

SUMMARY

Seven malignant esophageal tumors in dogs are described. In 6 of the 7 cases, the tumor actually or apparently occurred in relation to Spirocerca lesions.

The tumors in 4 cases occurred in the region of lesions containing the parasites. In 2 cases no parasites were noticed on postmortem examination. However, the presence of deformative ossifying spondylitis affecting the posterior thoracic vertebrae gave presumptive evidence of infection with Spirocerca. In 1 case the tumor was a surgical specimen mailed to the laboratory with no information pertaining to Spirocerca infection. All seven tumors occurred in the region of the esophagus where Spirocerca lesions characteristically occur.

Two of the tumors were fibrosarcomas, and five were osteosarcomas. Three of the osteosarcomas had metastasized. Hypertrophic pulmonary osteoarthropathy was found in association with the two fibrosarcomas and one of the osteosarcomas.

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ADDENDUM

Since preparation of the manuscript, 3 additional cases of canine malignant esophageal tumors have been encountered. One was a fibrosarcoma with metastases and was associated with deformative ossifying spondylitis, healed Spirocerca aneurysm of the aorta, and hypertrophic pulmonary osteoarthropathy. The second was an osteosarcoma associated with Spirocerca infection and hypertrophic pulmonary osteoarthropathy. The third was an osteosarcoma with metastasis and was associated with Spirocerca infection, deformative ossifying spondylitis, and hypertrophic pulmonary osteoarthropathy.

In Vitro Studies on the Anthelmintic Effect of Some Antimalarial Drugs

A. SHARAF, M.V.Sc., Ph.D., and I. M. SHIHATA, M.D.

Cairo, Egypt

CERTAIN antimalarial drugs have been found to have an anthelmintic effect.[1-10] The purpose of this investigation, therefore, is to study *in vitro*, the effect of three of the most common of these drugs, *viz.*, atabrine, paludrine, and camoquin, on the Taenia and Ascaris which infest the intestine of dogs, as well as to study their action on the intestinal motility.

MATERIALS AND METHODS

Tablets of atabrine hydrochloride 0.1 Gm. each, paludrine hydrochloride 0.1 Gm. each, and camoquin 0.2 Gm. each were used. Their solutions were freshly prepared by dissolving the tablets in Tyrode's solution and insoluble constituents were removed by filtration.

Intestinal worms (*Taenia pisiformis* and *Toxascaris leonina*) and strips of the small intestine were carefully taken from the intestine of freshly killed infested dogs and kept in Tyrode's solution in order to examine their response to the above drugs *in vitro*. A few mature segments of a Taenia were suspended in oxygenated Tyrode's solution at a constant temperature of 38.5 C. using a glass jar bath with an inner vessel of 50-ml. capacity. The same technique was also used for the Ascaris, using the whole worm or its upper part. Strips of the intestine, about 1 in. long, were also examined in the same way.

After recording the normal movement of Taenia, Ascaris, and intestine on a smoked paper, the effect of different doses of atabrine, paludrine, and camoquin was tested by the addition of their solutions to the organ bath.

RESULTS

Atabrine (table 1) had no effect on the Taenia in doses up to 0.2 mg. added to a 50-ml. bath but, with increased doses, consistent stimulation of the motility was recorded (fig. 1). No effect was noticed upon the Ascaris even in higher concentrations. Atabrine, moreover, produced relaxation in the intestinal muscles (fig. 2).

When camoquin (table 2) was used on Taenia, motility was stimulated with doses of 0.5 mg. or more (fig. 3). The spontaneous activity of the intestine was also increased when this drug was added in doses of 0.2 mg. or more (fig. 4). On the other hand, inhibition and diminution of the motility of Ascaris was obtained when the dose reached 5 mg. or more (fig. 5).

With paludrine (table 3), no effect was obtained on either the Taenia or Ascaris

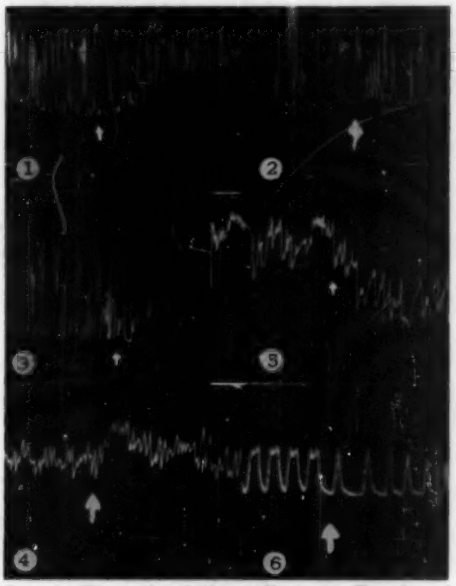

Fig. 1—The stimulating effect on Taenia of dogs of a 10-mg. dose of atabrine added to a 50-ml. bath.

Fig. 2—The inhibitory effect on a dog's intestine of a 5-mg. dose of atabrine added to a 50-ml. bath.

Fig. 3—The stimulating effect on Taenia of dogs of a 5-mg. dose of camoquin added to a 50-ml. bath.

Fig. 4—The stimulating effect on a dog's intestine of a 5-mg. dose of camoquin added to a 50-ml. bath.

Fig. 5—The inhibitory effect on Ascaris of dogs of a 30-mg. dose of camoquin added to a 50-ml. bath.

Fig. 6—The inhibitory effect on a dog's intestine of a 5-mg. dose of paludrine added to a 50-ml. bath.

From Cairo University, Cairo, Egypt.

TABLE 1—The Effect of Atabrine on Taenia, Ascaris, and Intestine of Dogs

Taenia		Ascaris		Intestine	
Dose/mg. added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect
0.1	No effect	0.2	No effect	0.2	No effect
0.2	No effect	0.5	No effect	0.5	Inhibition
0.5	Stimulation	1.0	No effect	1.0	Inhibition
1.0	Stimulation	5.0	No effect	2.0	Inhibition
5.0	Stimulation	10.0	No effect	5.0	Inhib.-relax.*
10.0	Stimulation	15.0	No effect	10.0	Inhib.-relax.
15.0	Stimulation	25.0	No effect	20.0	Inhib.-relax.
30.0	Stimulation	33.0	No effect	30.0	Inhib.-relax.

* Inhibition, then relaxation.

even when the dose was increased to 30 and 50 mg., respectively. Furthermore, an inhibitory effect was obtained on the intestine when paludrine was added to the bath at the rate of 1 mg. or more (fig. 6).

DISCUSSION

Since atabrine stimulates the activity of Taenia, this may explain why the parasite is eliminated alive after administration of this drug. Atabrine, therefore, is not a vermicide as has been recorded by Culbertson.[2] Because of atabrine's inhibitory influence on intestinal motility, the administration of a purge to help the elimination of the activated worms is suggested. However, the finding that atabrine has an inhibitory effect on the intestine does not correlate with the result of Sodman and Jung [9] and Shalash *et al.*[8] who reported that this drug possessed a purgative action. Apparently, atabrine can be used as an anthelmintic for tapeworms. While it does not kill the parasites, it is obnoxious to them and directly stimulates their musculature causing movements which detach their hold on the intestinal mucosa so that they are easily expelled by a subsequent purgative. The roundworm, *Toxascaris leonina*, is not affected *in vitro* and, therefore, would not be easily removed by atabrine *in vivo*.

Camoquin stimulates the motility of Taenia and causes movements which may, as in atabrine, detach the parasite's head from

TABLE 2—The Effect of Camoquin on Taenia, Ascaris, and Intestine of Dogs

Taenia		Ascaris		Intestine	
Dose/mg. added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect
0.1	No effect	0.2	No effect	0.1	No effect
0.2	No effect	0.3	No effect	0.2	Delayed stimulation
0.5	Stimulation	0.5	No effect	0.5	Delayed stimulation
1.0	Stimulation	1.0	No effect	1.0	Stimulation
2.0	Stimulation	2.0	No effect	5.0	Stimulation
3.0	Stimulation	5.0	Inhibition	10.0	Stimulation
4.0	Stimulation	10.0	Inhibition	20.0	Stimulation
5.0	Stimulation	20.0	Inhibition	30.0	Stimulation
10.0	Stimulation	30.0	Inhibition	50.0	Stimulation

TABLE 3—The Effect of Paludrine on Taenia, Ascaris, and Intestine of Dogs

Taenia		Ascaris		Intestine	
Dose/mg added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect	Dose/mg. added to 50-ml. bath	Effect
0.1	No effect	3.0	No effect	0.2	No effect
0.2	No effect	5.0	No effect	0.5	No effect
0.5	No effect	10.0	No effect	1.0	Inhibition
1.0	No effect	20.0	No effect	3.0	Inhibition
2.0	No effect	30.0	No effect	5.0	Inhibition
5.0	No effect	35.0	No effect	10.0	Inhibition
10.0	No effect	50.0	No effect	20.0	Inhib.-relax.*
20.0	No effect				
30.0	No effect				

* Inhibition, then relaxation.

the intestinal wall. It also stimulates intestinal motility and thus may probably cause the elimination of living parasites without the administration of a subsequent purgative. The effect on Ascaris, however, is inhibitory but larger doses are required to produce such action. Camoquin, therefore, may act as a vermicide and may also eliminate the parasite by the excessive intestinal movements which it causes. A purgative may also be given subsequently.

Paludrine, on the other hand, even in large doses, produces practically no effect on either Taenia or Ascaris. From this preliminary pharmacological study paludrine, therefore, can not be recommended as an anthelmintic agent against these two parasites.

Summary and Conclusions

1) Atabrine produces a stimulating effect on the canine Taenia, no effect on the Ascaris, and an inhibitory action on the canine intestine. It may be used, with a purge, as a taeniafuge.

2) Camoquin has a stimulating action on both the Taenia and the intestine of dogs and, in large doses, it inhibits the movement of Ascaris. Therefore, it may be used as a vermifuge with or without a purge.

3) Paludrine has proved to be ineffective against either Taenia or Ascaris; therefore, it is of no importance as a canine anthelmintic.

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Continuous Phenothiazine Therapy for Horses
II. A Taxonomic Study Following Four Years of Treatment

J. H. DRUDGE, D.V.M., Sc.D.; Z. N. WYANT, A.M.; G. W. ELAM

Lexington, Kentucky

Literature Cited

In the early studies [6, 15, 17, 23, 24] on the use of small repeated doses of phenothiazine in the horse, fecal egg counts were utilized as the essential means of evaluating therapeutic action. In view of the already established effectiveness [12, 14, 19] of single therapeutic doses of phenothiazine for the removal of strongyles, the marked reduction of egg counts following small repeated doses suggested a corresponding removal of the mature strongyles from the intestinal tract. However, the work of Gibson [10, 11] indicated that the action of 1.0-Gm. daily doses of phenothiazine was inhibiting egg production but not removing the strongyles. In contrast with this, more recent workers [1, 21, 22] concluded from their investigations, which included postmortem examinations, that 2.0-Gm. daily doses of phenothiazine were removing the strongyles as indicated by the reduction of the eggs in the feces.

An earlier report [3] by the present authors, dealing with the general effects observed during the fourth year of a long-term study of low-level phenothiazine administration, tended to agree with Gibson. It associated continued low or negative strongyle egg counts on the feces with the recovery of relatively large numbers of both large and small strongyles from the cecums and large colons of treated mares which were destroyed at the close of the treatment period. Those treated, however, were found to be harboring fewer small strongyles than the control animals. Thus, it appeared of further interest to compare the species of small strongyles and other parasites remaining in the two groups of animals to provide information relative to possible differences among species in their sensitivity to the low-level type of phenothiazine administration.

Materials

The experimental set-up and treatment of the animals were outlined earlier [3] along with the method of sampling for the collection of the parasites. For identification, the worms were cleared in beechwood creosote and examined individually with a compound microscope.

Results

The various species of parasites recovered from the gastrointestinal tracts of 2 treated and 2 untreated mares are recorded in table 1. In general, the parasite fauna in both groups were composed of the same species.

Two species of bots *(Gastrophilus intestinalis* and *Gastrophilus nasalis)* were present in about equal numbers in both groups. The stomach worm *(Habronema muscae)* was also found in all but control mare 2 although the infections in the treated animals were heavier. Microscopic examination of the female *H. muscae* revealed the presence of the typical large number of vermiform embryos *in utero* in all but a very few specimens from both treated animals as well as the control mare 1.

The minute stomach worm *(Trichostrongylus axei)* was recovered from the 7-year-old mare only. This infection was of a low order, but 67 of 69 female specimens of this species showed eggs *in utero.*

In the small intestine, only one ascarid *(Parascaris equorum)* was found, a mature female worm from the 12-year-old treated mare.

Three species of large strongyles *(Strongylus vulgaris, Strongylus edentatus,* and *Strongylus equinus)* were in the cecal and large intestinal collections from each group. The 7-year-old treated mare showed the heaviest large strongyle infection, while no large strongyles were recovered from control mare 1. None of the large strongyle females from either of the treated animals was observed to contain eggs *in utero,* whereas eggs were readily detected in all but 1 *S. edentatus* and 2 *S. vulgaris* females in the large strongyle collections from the control mare 2. No consistent trend was noted in the sex ratios of the large strongyles from the treated animals.

The species of small strongyles (Cylicostomes) were arranged into three groups in the table. The first group, the species common to both treated and untreated animals, comprised the bulk of the small strongyle infections recovered. Within this group of small strongyles, no genus or species was consistently dominant in the various animals irrespective of treatment. The second and third groups of small strongyles, consisting of the species recov-

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

ered only from the untreated animals or only from the treated animals, respectively, were represented by relatively small numbers.

Immature forms (fourth-stage larvae) accounted for nearly one third of the small strongyles recovered from the 12-year-old treated mare while in the other treated and the control mares about 10 per cent were immature.

The adult female small strongyles from the two groups of animals were compared as to the presence of eggs *in utero*. Eggs were present in 60.7 per cent of the females from control mare 1 and in 88.4 per cent from the control mare 2. The relatively small proportion in the former was largely due to the female *Cylicostephanus minutus, Cylicostephanus longibursatus,* and *Cylicostephanus calicatus* of which 26.5, 38.5, and 60.9 per cent, respectively, contained eggs *in utero*. In the remaining species, above 85 per cent of the females contained eggs. From control mare 2, 85.7 per cent of the female *C. minutus* and 80.0 per cent of the female *C. calicatus* collected showed eggs *in utero* but no *C. longibursatus* females were recovered.

In contrast, only a few of the small strongyles taken from the treated mares had eggs *in utero*, the percentage of females showing eggs being 1.4 from the 12-year mare and 7.8 from the 7-year mare. Only 1 of 50 female *Cylicocercus catinatus* from the former, and 5 of 55 female *C. catinatus* and 3 of 6 *Cylicostephanus calicatus* from the latter were observed to contain eggs.

The ratios of female/male small strongyles from the treated and control animals were also compared. As a group, the small strongyles collected from the treated mares were male-deficient, whereas those taken from the control animals more closely approximated the theoretical 1/1 sex ratio. A statistical comparison of this relationship by the X^2 test revealed a significance beyond the 1 per cent level. For this test, the average proportion of females to males from the control animals was employed as the expected relationship.

The analysis was extended to test the sex relationships in the species making up the small strongyle infections in the treated animals. For the 12-year mare, only *Cylicostephanus minutus* failed to show a sig-

TABLE 1—Gastrointestinal Parasites in Phenothiazine-Treated (2.0 Gm. Daily) and Untreated (Control) Mares at the Close of the Fourth Year

Organ	Parasite	No. of parasites			
		Treated mare		Untreated mare	
		12-year-old	7-year-old	Control 1	Control 2
Stomach	*Gastrophilus intestinalis*	58	97	59	99
	Gastrophilus nasalis	157	58	56	51
	Habronema muscae	516	1,116	273	0
	Trichostrongylus axei	0	93	0	0
Small intestine					
	Parascaris equorum	1	0	0	0
Cecum and large intestine					
Large Strongyles					
	Strongylus vulgaris	22	527	0	102
	Strongylus edentatus	0	63	0	29
	Strongylus equinus	0	2	0	9
Small Strongyles (Cylicostomes)					
	Cyathostomum coronatum	0	70	1,590	100
	Cylicocercus catinatus	3,285	4,375	3,640	26,775
	Cylicocyclus nassatus	0	1,200	19,490	0
	Cylicocyclus leptostomus	0	375	5,210	75
	Cylicostephanus calicatus	0	515	1,340	425
	Cylicostephanus minutus	540	675	13,870	750
	Cylicostephanus longibursatus	1,060	850	13,860	75
	Cyathostomum labratum	0	0	50	0
	Cylicocyclus insigne	0	0	0	75
	Cylicodontophorus bicoronatus	0	0	50	75
	Gyalocephalus capitatus	0	0	0	25
	Cyathostomum labiatum	0	150	0	0
	Cylicostephanus poculatus	0	65	0	0
	Immature Cylicostomes (No.)	2,215	900	6,650	2,775
	Immature Cylicostomes (%)	31.2	9.8	10.1	8.9
	% ♀ with eggs (Cyclicostomes)	1.4	7.8	60.7	88.4
	Ratio ♀/♂ (Cylicostomes)	5.4:1	6.8:1	1.6:1	1.2:1
Other					
	Oxyuris equi (immature)	665	25,425	360	600
	Probstmayria vivipara	+	+	—	—

nificant difference; in the other mare, *C. calicatus, C. minutus, C. longibursatus,* and *Cylicocylus leptostomus* showed no significant differences in the sex ratios. The observed ratio deviations in *Cylicocercus catinatus* and *Cylicocyclus nassatus* from the latter mare were significant beyond the 2 per cent level.

Mature pinworms *(Oxyuris equi)* were not observed in any of the mares of this study. However, immature larval forms of this species were present in all 4 animals, the 7-year-old treated mare carrying a comparatively heavy infection of this species.

The minute pinworm *(Probstmayria vivipara)* was found in both treated but in neither control animal. No attempt was made to estimate the magnitude of the infections in the treated mares. Microscopic examination of female specimens from both mares revealed various stages of embryo development *in utero.*

DISCUSSION

All of the species of parasites encountered in this study had been previously recorded [2, 7, 20] as infecting horses in this country.

The observations on the strongyles were of particular interest. In comparison with other reports,[5-7, 20] they were quite typical. Three species of large strongyles were recovered from both treated and untreated animals. The bulk of the small strongyle infections in both groups was made up of the same species, with the treated harboring lighter infections than the control mares. These determinations failed to demonstrate relative differences in susceptibility to this drug by the species of strongyles encountered. However, pasture conditions permitted the cross transmission of the strongyles between treated and untreated animals. Unless the drug prevented development of ingested infective larvae, manifestations of differences should not be expected.

In the present observations, the presence of eggs *in utero* in the strongyle females appeared to offer an index of susceptibility to this type of phenothiazine administration. Most of both large and small strongyle females from untreated mares were observed to contain eggs. In contrast, only a small percentage of the small strongyle females from the treated mares showed eggs *in utero;* of the nine species, only two *(Cylicocercus catinatus* and *Cylicostephanus calicatus)* presented specimens with eggs. This suggests that at least certain species of small strongyles are capable of carrying on reproductive functions in the presence of the phenothiazine, which in effect may be regarded as an index of tolerance to drug action.

In view of the foregoing, the sex ratio relationships shown by the small strongyles assume added interest. Theoretically, the failure of the females to contain eggs could be due to direct action of the drug on the females and/or indirectly through the males. The collections of small strongyles from both treated animals were characterized by a significant deficiency of male forms. Of the species observed with eggs *in utero, Cylicostephanus calicatus* also failed to show a shortage of males. Within the same genus, *C. minutus* and *C. longibursatus* presented, along with *Cylicocyclus leptostomus,* sex ratios not significantly different from those for the respective species in the control animals. None of the females of these three species were observed with eggs *in utero;* however, even in the control mares, both *Cylicostephanus minutus* and *C. longibursatus* appeared to be relatively poor egg layers. In contrast, *Cylicocercus catinatus,* which also revealed females with eggs, showed a significant deficiency of males in the treated animals.

Although none of the large strongyle females from the treated animals were observed to contain eggs *in utero,* a change in the sex ratios was not evidenced.

These sex-linked observations are not regarded as sufficient to demonstrate, conclusively, relative refractoriness to drug action among the strongyle species encountered. Nonetheless, such relationships are strongly suggested, and additional observations are being made. Such information would appear to be a prerequisite to an evaluation of the acquisition of phenothiazine resistance among these forms. Since the treated animals were under continuous exposure to the strongyles infecting the control animals in this study, the observations could not be interpreted as an index of an acquired resistance by the strongyles to phenothiazine.

Trichostrongylus axei, which is rather closely related to the strongyles, was recovered from the stomach of 1 of the treated animals. The findings of various work-

ers [14, 19] are not in agreement as to the effectivness of therapeutic doses of phenothiazine in the removal of *T. axei* from the horse; however, in the sheep and cattle hosts, this parasite was found [13, 16, 18, 19] to be sensitive to therapeutic doses. The present observations indicate that the low-level type of administration is not effective against mature *T. axei* in the horse. Possible action against the free-living, preparasitic larval stages of this species was not excluded.

No action against the stomach worm *Habronema muscae* was detected in the present study. This appears to be in accord with previous reports.[9, 12]

A relatively heavy infection of immature pinworms, *Oxyuris equi*, in 1 of the treated animals, suggests an inhibitory effect by the drug. This would tend to agree in part with the previous report [4] that immature *O. equi* are more sensitive than mature pinworms to phenothiazine in therapeutic doses. However, according to Wetzel [25] a high percentage of *O. equi* may be immature in untreated horses. Thus it is felt that no definite action by the low-level system of phenothiazine administration was established in the present observations. Likewise, no effect on the minute pinworm, *Probstmayria vivipara*, was detected.

SUMMARY

A taxonomic analysis was made of the parasite fauna obtained in horses following four years of low-level phenothiazine administration. The species of parasites encountered were essentially the same in both treated and control animals. The nine species represented in the small strongyle collections from treated mares were characterized by only two species with females showing eggs *in utero*, and by male forms being generally deficient. These observations suggested relative refractoriness to drug action by certain species of small strongyles. The related strongyloid *(Trichostrongylus axei)* also appeared to be drug-tolerant. No action against the bots *(Gastrophilus intestinalis* and *G. nasalis)*, the stomach worm *(Habronema muscae)*, and the pinworms *(Oxyuris equi* and *Probstmayria vivipara)* was detected in these observations.

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Antibody Response of Horses Following Intravenous Injection of Small Amounts of Active Japanese B Encephalitis Virus After Recent Natural Infection

KENNETH F. BURNS, Lt. Colonel, V.C., U.S. Army

Fort Sam Houston, Texas

A HIGH incidence of neutralizing antibodies against the virus of Japanese B encephalitis has been observed among horses and human beings in endemic areas of Japan.[1-3] This is believed to be due to widespread inapparent infection with the virus. The geographic distribution of the neutralizing antibodies coincides with that of the serologically confirmed cases of Japanese B encephalitis. Further, Kii *et al.*[4] demonstrated that horses developed antibodies after a proved viremia.

A high incidence of positive complement-fixation tests was found in horses during the 1947 epizootic in Japan.[5] By periodical testing of several groups of horses which exhibited no signs of disease in epidemic areas, the change from negative to positive complement-fixing titers was observed, as well as the development of neutralizing antibodies, during the epidemic season.[6] The duration of complement-fixing antibodies in the equine blood stream was limited to two or three months. It therefore appeared desirable to study the antibody response in experimentally infected animals, to provide a proper basis for interpretation of the results observed in the field.

METHODS AND MATERIALS

Horses.—Twenty-four young (1 to 2 years old) horses were obtained from Hokkaido (Kitami), the northernmost island of the Japanese archipelago, for this project. Prior to 1948, this island had been considered as a nonepidemic and nonepizootic area. These animals were procured from an area in Hokkaido where no cases were reported during the 1948 outbreak and, following importation to Honshu, were stabled in Yoyogi, a suburban area of Tokyo.

For inoculation purposes, two strains of Japanese B encephalitis virus were employed: (1) an Okinawa strain, which was isolated from a fatal human case in 1949, and propagated through five consecutive intracerebral mouse passages, and (2) the Nakayama strain, which is widely employed as a standard strain of Japanese B virus and has had numerous mouse passages since its original isolation in Japan. For the inoculation of horses, suspensions of infected mouse brain of known $l.d._{50}$ titers were employed. The infected mouse brain material was titered for virulence prior to inoculation and stored in a dry ice chest (—50 C.) until the titration was completed, at which time it was immediately utilized.

Blood samples were collected in the usual manner, and the serums were stored in a dry ice chest.

Neutralization Tests.—Intracerebral neutralization tests were carried out in mice. Mixtures of 0.2 ml. of undiluted and unheated serum and 0.2 ml. of three or four tenfold dilutions of mouse brain suspension infected with the virus of Japanese B encephalitis (Nakayama) were incubated for two hours in a water bath at 37 C. White mice, weighing approximately 8 Gm., were then inoculated intracerebrally with 0.03-ml. amounts of each of the serum-virus mixtures. Four or 5 mice were used per dilution of virus suspension. The inoculated mice were observed for two weeks. A normal rabbit serum known to be negative for Japanese B virus was included as a control for each test.

The neutralizing capacity is expressed by the neutralization log index. (The difference in log dilutions of $l.d._{50}$ end points of virus-serum mixture containing the serum in question and the negative serum control.) No serum was regarded as positive unless the neutralization log index was 1.7 or greater. Those less than 1.0 are negative, and results between 1.0 and 1.7 are expressed as equivocal.

Complement-Fixation Tests.—Our technique was similar to that outlined by Casals and Palacios[7] with overnight incubation of serum-antigen-complement mixtures and complement titrations in the presence of antigens at 5 C. The antigens were prepared from the Nakayama strain by the benzol extraction method described by Espana and Hammon.[8] A control antigen was prepared from normal mouse brain by the same method. The titer of a serum is the highest original dilution showing 2+ (approximately 50%) or greater fixation.

RESULTS

Changes of Neutralization and Complement-Fixation Titers of Horses from July

From the Fourth Army Area Medical Laboratory, Brooke Army Medical Center, Fort Sam Houston, Texas.

The research required for this article was conducted at the 406th Medical General Laboratory, Tokyo, Japan, under the command of Colonel Robert L. Hullinghorst, M. C., U. S. Army.

through December 1949.—Monthly bleedings were conducted on the 24 horses, stabled at Yoyogi, a residential district in Tokyo, Japan, for neutralization and complement-fixation tests. It was anticipated that in this area an opportunity would be afforded for transmission of the disease in nature by insects. Results are shown in table 1 which are representative of serological changes that occurred in the horses at Yoyogi.

Nineteen of the horses had negative neutralizing log indexes (NLI) during July (log index less than 1.0), while animals 5, 13, 16, 20, and 24 had neutralization log indexes of 1.1, 1.1, 1.6, 2.6, and 1.2, respectively. Blood specimens taken again during the month of August were negative on horses 5, 13, and 16. Animal 24 had a log index of 1.3 during August with animal 20 showing a positive neutralization log index (NLI 1.7 or greater) prior to the expected seasonal appearance of the disease in the Tokyo area. Thus, with only 2 exceptions (No. 20 positive, No. 24 equivocal [NLI >1.0 but <1.7]) all animals were negative for neutralizing antibodies up to and including August; however, by September 30 all horses exhibited a positive neutralization log index. Serum specimens obtained from these animals during October, November, and December all had high titers.

Complement-fixation tests on serums obtained in July and August from these animals were all negative with 15 of the animals developing low complement-fixing titers (1:4 to 1:16) during September. All but 5 of these animals became negative again during December. These 5 animals, whose positive complement-fixing titers persisted, were of a very low titer (1:2 to 1:4). None of the horses exhibited clinical manifestations of encephalitis during the period of these observations. Horse 22 was destroyed for autopsy in December, and neither macroscopic nor microscopic changes were observed.

Changes of Neutralization and Complement-Fixation Titers of Horses Following Experimental Inoculation with Japanese B Encephalitis Virus.—Two groups (I and II) of 10 horses each (1-10 and 11-20) were inoculated intravenously with 3.0 ml. each of $10^{-6.2}$ and $10^{-5.2}$ dilutions, respectively, of mouse brains infected with the Okinawa strain which had a $l.d._{50}$ titer in mice of $10^{-5.7}$ per 0.03 ml. Horse 21 was injected intravenously with 3.0 ml. of $10^{-7.5}$ concentration of the virus suspension of the Nakayama strain. The intracerebral titer of this virus suspension was $10^{-8.3}$ per 0.03 ml. in mice. The remaining 2 horses (23 and 24) acted as controls and did not receive any inoculation.

On Jan. 18, 1950, just prior to inoculation, blood specimens were drawn with additional serial serum specimens obtained during the ensuing weeks to include February 14. In order to determine the presence and duration of a viremia in the infected horses, blood was collected in the normal manner, the serum was allowed to separate and was immediately inoculated intracerebrally into mice after serially diluting with physiological saline to which 10 per cent normal rabbit serum was added.

At no time was the presence of circulating virus in the blood stream demonstrated. The representative results of neutraliza-

TABLE 1—Neutralization and Complement-Fixation Titers from Representative Horses Stabled at Yoyogi Through Epidemic Season

Horse (No.)	Date horses were bled													
	July 8		Aug. 11		Aug. 25		Sept. 30		Oct. 25		Nov. 25		Dec. 20	
	N	C	N	C	N	C	N	C	N	C	N	C	N	C
1	0.5	0	0.6	0	....	..	4.1	1:16	4.1	1:16	4.3	0	3.7	0
4	0.3	0	0.7	0	....	..	3.4	0	4.2	0	3.4	0	3.7	0
7	0.5	0	0.3	0	....	..	3.9	1:8	4.1	1:4	4.5	1:4	4.0	0
8	0.5	0	0.7	0	....	..	3.9	1:8	3.6	1:8	4.7	1:8	3.7	1:4
11	0.9	0	0.3	0	....	..	3.9	1:4	4.4	1:4	4.9	0	3.9	0
14	0.8	0	0.3	0	....	..	3.9	1:4	4.1	1:4	4.5	1:4	3.6	1:4
17	0.8	..	....	..	1.5	0	4.1	1:4	4.6	1:4	4.4	0	3.7	0
18	0.8	0	....	..	0.9	0	2.6	0	3.9	0	4.1	0	3.9	0
20	2.6	0	....	..	1.9	0	3.6	1:8	4.3	1:4	4.8	1:4	3.7	1:4
22	0.8	0	....	..	1.3	0	4.1	1:8	4.5	1:8	5.2	1:4	Autopsied	
23	0.8	0	....	..	1.6	0	2.8	0	3.7	0	3.8	0	3.7	0
24	1.2	0	....	..	1.3	0	3.8	1:4	3.9	0	4.8	0	4.2	0

N—neutralization log index; C—complement fixation titer.

tion and complement-fixation tests on these serum specimens are shown in table 2.

The 2 control animals (23 and 24) remained negative for complement-fixing antibodies during the period of observation. In contrast, all animals which received an intravenous inoculation of the virus subsequently developed complement-fixing antibodies. Five horses had a complement-fixation titer of 1:4 prior to inoculation, and subsequently showed a definite increase following this antigenic stimulation. Of the 7 animals in group 1 which were negative prior to inoculation, 5 and 2 became positive in six and eight days, respectively; while in group 2, 7 animals (1 and 6) developed complement-fixing antibodies in three and six days, respectively. All animals but 1 which had received a virus inoculation remained positive during the subsequent four-week period of observation. The 1 exception, horse 3, developed a titer of 1:4 in three days and was negative on the twelfth day thereafter.

The highest complement-fixation titers which individual animals developed, together with frequency distribution, are shown in table 3. As portrayed, the animals of group 2 developed a higher titer than those of group 1. The difference in titers would appear to be significant.

DISCUSSION

In accordance with previous observations,[4-6] horses stabled in Tokyo during the seasonal appearance of Japanese B encephalitis developed neutralizing antibodies during the month of September, 1949. Simultaneously, the majority of these horses became positive by complement-fixation tests. It is noteworthy that all of the horses developed a positive neutralization log index while 16 of 24 animals (67 %) produced complement-fixing antibodies. In contrast to the neutralization log index which was still at a high level in December, 1949, the complement-fixation titer decreased during the same period to a negligible level. This confirms the results of a previous study.[6] Subsequently, these horses (groups 1 and 2) were inoculated with 32 and 320 mouse $l.d._{50}$, respectively, of the Okinawa strain of Japanese B encephalitis virus, and 1 animal received 630 mouse $l.d._{50}$ of the Nakayama strain.

The most probable mode of infection of Japanese B encephalitis is considered to be an arthropod with the mosquito being the most frequently mentioned vector. Assuming that the mosquito is responsible for the natural infection of horses, an attempt was made to estimate the dose which might be inoculated through a mosquito bite. Of

TABLE 2—Neutralization and Complement-Fixation Titers from Representative Horses Stabled at Yoyogi After Inoculation with Japanese B Virus on January 18, 1950

Group	Horse (No.)	Date horses were bled: January 18	January 24	January 25	February 1	February 7	February 14
1	1	1:4*	1:4	1:16	1:8	1:16	—
		4.1**	4.1	3.5	>4.7	>4.6	4.5
	4	0	0	1:8	1:16	1:16	1:16
		2.7	3.3	3.9	>4.8	>4.6	3.8
	7	0	1:4	1:8	1:8	1:8	1:16
		4.4	3.7	3.5	4.6	4.2	—
	8	1:4	1:16	1:16	1:16	1:32	1:16
		4.6	3.0	3.9	4.8	>4.6	4.3
2	11	0	1:8	1:16	1:32	1:32	1:16
		4.5	>4.1	>4.1	>4.8	>4.6	—
	14	1:4	1:16	1:16	1:64	1:128	1:32
		>4.8	3.9	3.9	>4.8	4.6	—
	17	0	1:16	1:16	1:64	1:128	1:64
		4.4	>4.1	>4.1	>4.8	>4.6	—
	18	0	1:16	1:16	1:32	1:32	1:16
		4.8	3.7	3.9	>4.8	>4.6	4.1
	20	1:8	1:16	1:16	1:64	1:128	1:32
		4.4	3.7	3.7	>4.8	>4.6	4.9
3	21	0	1:16	1:16	—	1:64	1:32
	(Nakayama virus)	3.7	>4.1	3.9	4.6	>4.6	>4.1
	23	0	0	0	0	0	0
	(Control-no virus)	3.6	3.8	3.8	4.6	3.8	2.7
	24	0	0	0	0	0	0
	(Control-no virus)	3.7	4.0	4.2	4.6	4.2	4.2

* Complement fixation titer; ** neutralization log index; — test not conducted.

necessity there are a number of factors which must be taken into consideration; the titer of the virus which a mosquito harbors in nature, the amount of fluid injected into the victims by the bite of a mosquito,

TABLE 3—Frequency Distribution of the Highest Complement-Fixing Titers that Individual Animals Developed

Group	Dilutions*						
	4	8	16	32	64	128	Total
1	1	0	6	3	0	0	10
2	0	0	1	2	3	4	10

* Reciprocal of the dilution.

the number of mosquitoes per animal, et cetera. Apparently, no adequate study has been reported which takes into consideration all of the above mentioned factors. Therefore, on the basis of estimates for the variables, a virus dose was selected equivalent to between $10^{1.5}$ and $10^{2.5}$ mouse $l.d._{50}$. The virus suspension of the Okinawa strain had an intracerebral titer in mice of 0.03 ml. of $10^{-5.7}$ of the original brain. Therefore, 3.0 ml. of $10^{-6.2}$ and $10^{-5.2}$ dilutions of the original mouse brain is equivalent, respectively, to $10^{1.5}$ and $10^{2.5}$ mouse $l.d._{50}$.

There was no unusual increase in neutralization log indexes following inoculation with the virus, only a few of the animals demonstrating an increased titer. This may be explained by the fact that all animals had comparatively high (2.7-4.9) neutralization log indexes prior to inoculation. Two animals (4 and 12) which had relatively low neutralizing titers prior to inoculation demonstrated a significant increase to the virus of Japanese B encephalitis.

All animals which received viral inoculations developed complement-fixing antibodies. In a previous study,[8] 1 horse which was stabled in Okayama prefecture, and had a positive neutralization index but a negative complement-fixing titer prior to the epidemic season of Japanese B encephalitis of 1947, developed a positive complement-fixing titer during the summer months. Identical results have been obtained in the present study; thus, horse 20, which had a positive neutralization but a negative complement-fixing titer prior to the epidemic season, showed a complement-fixation titer of 1:8 during September. These data indicate that during the epidemic season complement-fixing antibodies may develop in immune as well as nonimmune horses. This conclusion is further supported by the fact that 86 per cent of 69 normal horses developed complement-fixing antibodies during the summer months of 1947.[1] From these observations, it is quite evident that immune horses develop complement-fixing antibodies during the seasonal appearance of the disease. This perhaps is the result of a recurrent clinically inapparent infection. Such an opinion is supported by data of the present experiment in which immune horses developed complement-fixing antibodies without any encephalitic manifestations following an intravenous inoculation with virus. Although the presence of a viremia was not demonstrated in any of the horses, this does not necessarily mean that it was nonexistent. Cognizance must be given to the presence and concentrations of neutralizing antibodies.

It is noteworthy that the development of positive complement-fixing antibodies or of titer increase occurred rather rapidly (in 3 to 8 days) following inoculation with the virus. In contrast with these results, a relatively slow appearance of complement-fixing antibodies was obtained in horses which were not immune and had been inoculated with Japanese B virus.[9] Thus, 1 horse, which received an intracerebral dose of 5 ml. of 10 per cent mouse brain emulsion infected with strain 2869 of Japanese B virus, developed complement-fixing antibodies in eight days, while 2 other horses, which received an identical inoculation of two other strains (2683 and Nakayama) of Japanese B virus, did not develop complement-fixing antibodies during an observation period of nine and seven days, respectively. Similar results were reported by Sabin *et al.*[10] in human beings following inoculation with Japanese B vaccine. These facts may be explained by a booster response.

SUMMARY

Twenty-four horses which were stabled in Tokyo (22, 1, and 1 had negative, equivocal, and positive neutralization indexes, respectively, for the virus of Japanese B encephalitis) developed neutralizing antibodies or demonstrated an increase in titer during the seasonal appearance of the dis-

ease among the human population without any apparent evidence of illness in Equidae.

Fifteen of these animals developed low titer complement-fixing antibodies (1:4 to 1:16) during September, 1949. By December, all animals had negligible titers (1:2 to 1:4), indicating the transitory nature of complement-fixing antibodies for Japanese B virus in horses.

On Jan. 18, 1950, these horses received an intravenous inoculation of (32 to 320 mouse $l.d._{50}$) Japanese B encephalitis virus and again developed complement-fixing antibodies. This response has been attributed to a booster type of reaction. Two animals showed an increase in neutralizing antibody titer. A majority of them did not demonstrate a titer rise probably because of the maximal neutralization titer developed prior to intravenous inoculation. None of the inoculated animals exhibited clinical manifestations associated with any encephalitic infection during the period of these observations.

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The Pathogenesis of Telangiectasis in the Bovine Liver
I. The Incidence of Telangiectasis

A. C. ANDERSEN, V.M.D., Ph.D.

Davis, California

DURING the past four decades or more a number of veterinary pathologists have mentioned the finding of blood spaces, now recognized as telangiectasis, in the parenchyma of the bovine liver. The condition is of dubious clinical significance, but it is of great economic importance to the meat industry because of the condemnation of affected livers for esthetic reasons in meat inspection.

Telangiectasis is recognized in the bovine liver as blood-filled spaces resulting from the displacement of hepatic parenchyma. These lesions may be microscopic in size or visible to the unaided eye as purplish foci up to 2 cm. in diameter. Microscopically, the lesion is seen to occupy an area varying from a part of a lobule to a confluent focus occupying parts of several contiguous lobules.

The lack of knowledge regarding the causative factors responsible for these lesions has left a maze of confusing descriptive terms in veterinary literature. It is interesting to observe the changes in terminology through the past years, because these descriptive terms direct attention toward an etiological factor.

In 1906, Kitt [1] described the lesions as capillary telangiectases due to passive hyperemia from rumen pressure in tympanites. The 1913 edition of Hutyra and Marek [2] described the condition as angiomata, using the terms hemangioma cavernosum or telangiectasis capillaris. The later edition by the same authors [3] used a more descriptive terminology, namely, multiple hemangioma cavernosum or telangiectasis capillaris et hemorrhagica maculosa. Joest [4] discussed similar lesions as hemangioma of capillary origin, designating the lesions as angioma simplex or "teleangiecktasia" and angioma cavernosum (sc. capillare). Telangiectasis was formerly mentioned by Runnells [5] under tumors as cavernous angioma. In 1932, Feldman [6] in reviewing this problem stated, "Telangiectasis is not uncommonly encountered in the liver of cattle, especially old cows, and is designated by different writers as capillary telangiectasis, angiomatosis, cavernous angioma, spotted capillary angiomatosis, and multiple disseminated angioma of the liver; occasionally it has been erroneously grouped with the genuine neoplasms, as noted in the older literature." A similar discussion is given by Jackson.[7] Apparently, telangiectasis is discussed by Gaiger and Davis [8] as cavernous angioma and is not clearly distinguished from the descriptions of cavernous angioma given by Feldman,[6] Harvey, Dawson, and Innes,[9] and Ogilvie.[10] More recent literature proposes hepatic cell necrosis, an inflammatory process or both as the cause of telangiectasis.[11-18]

Because of the existing confusion and lack of information, the work reported in this study was undertaken in an effort to clarify the etiology and pathogenesis of telangiectasis in the bovine liver. The material will be presented in sections covering (I) the incidence of telangiectasis, (II) the morphology of the lesion, (III) the question of a microbial etiological agent, (IV) a possible formation mechanism, and (V) a comprehensive discussion.

INCIDENCE OF TELANGIECTASIS IN THE BOVINE LIVER

It has long been recognized that there are variations in both size and severity of telangiectatic lesions occurring in the bovine liver. This is exemplified by such terms as multiple cavernous or spotted capillary angiomatosis, used in previous veterinary literature to describe variations in the gross appearance of these lesions. Several authors refer to the lesions as common or numerous in the bovine liver and describe them as varying greatly in size.[2, 6, 12]

In 1953, the Meat Inspection Division of the United States Bureau of Animal Industry listed the loss of 272,063 cattle livers and 774 calf livers because of telangiectasis.[14] This record includes only those livers condemned because of telangiectasis and does not include livers with lesions too small or too few for condemnation. Nevertheless, this represents 13.89 per cent of all cattle livers

From the School of Veterinary Medicine, University of California, Davis. Submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy.

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Ante- and postmortem information was made available by the courteous help of the United States meat inspectors.

and 1.23 per cent of all calf livers condemned for human consumption. Cohrs[15] reported an incidence between 7.5 and 9.6 per cent in slaughtered cattle in Germany.

Other records show a much higher incidence when cattle are placed in the feedlot for fattening. Robinson, Jasper, and Guilbert[16] found the incidence of telangiectasis to be 81.0 per cent in cattle in the feedlot for fifty-six days, declining to 2.4 per cent at 160 days. Jensen *et al.*[17] found the incidence of telangiectasis to be 52.0 per cent on the eightieth day in the feedlot in one experiment, and 40.0 per cent on the seventy-seventh day in another. In the latter experiment, these authors found that the incidence of telangiectasis decreased after the seventy-seventh day to 0.0 per cent by 120 days and rose to 10.0 per cent after 167 days.

These reports indicate a lack of correct data as to the real incidence of telangiectasis in the livers of cattle. Therefore, study has been undertaken to obtain information as to the actual presence of all visible lesions in this organ.

It is well known that telangiectasis occurs in the livers of cattle, whether used for beef, dairy, or work purposes.* In the search for an etiological factor responsible for this condition, several questions arise as to the role played by the sex, age, and husbandry of the animal. To provide such information, a study was made of 1,570 bovine livers at an abattoir. Data on age and sex were recorded as well as an estimate of the severity of telangiectasis. In addition, the livers were classified as to whether they were passed or condemned for human consumption. The cases were not selected, being those available by chance on the days when the survey was made.

The lesions recorded in these data were recognized in the fresh liver as dark brown areas filled with blood, in contrast to the lighter brown or tawny color of the normal organ. The lesions are more readily observed in the fresh liver, as this organ becomes darker upon exposure to the air, which reduces the contrast in color for an accurate diagnosis.

The method of examination of the livers was as follows:

Each liver was removed from the pluck tray immediately following the examination made by the U. S. meat inspector. The organ was washed in running water to remove surface blood and held in the light to examine all surfaces for the presence of telangiectasis. Portions of the organ were incised when the severity of lesions was in question or for the removal of tissue for microscopic examination. Several hundred pieces of liver tissue were placed in fixatives for verification of the diagnosis. No doubtful lesions, such as bruises or traumatic injuries, engorged blood

* Personal communication with Dr. Livio G. Raggi, formerly assistant professor in animal pathology at the School of Veterinary Medicine, Perugia, Italy, revealed the presence of this condition in cattle used for work purposes.

Fig. 1—Cut surface of the liver from a 3-year-old cow showing lesions of telangiectasis. One-half normal size.

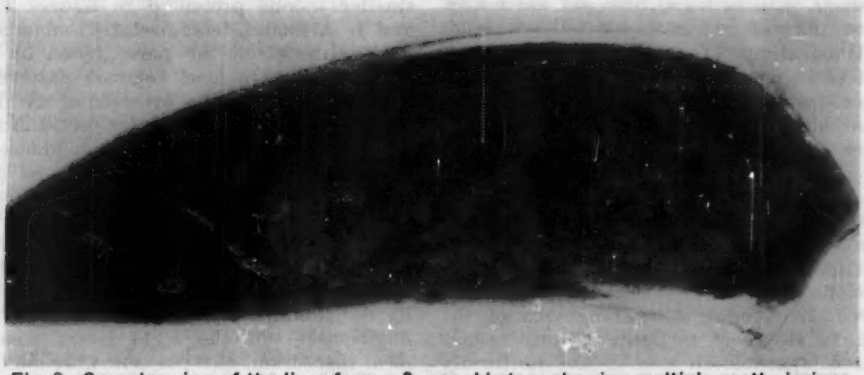


Fig. 2—Capsular view of the liver from a 2-year-old steer showing multiple spotty lesions of telangiectasis. One-half normal size.

Fig. 3—Cut surface of the liver shown in figure 2. Notice the smaller lesions of telangiectasis; the distribution of the lesions is similar to those shown in figure 1. One-half normal size.

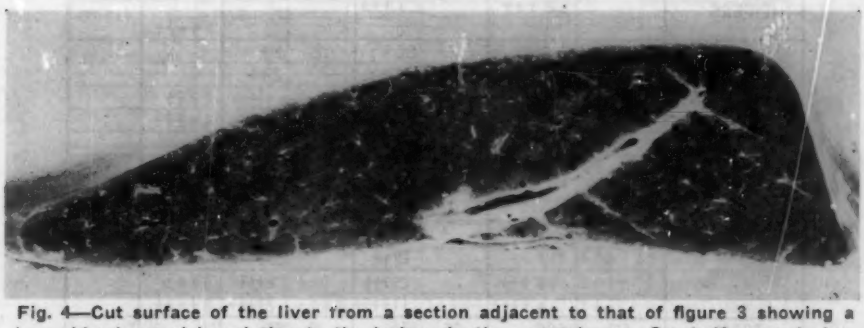


Fig. 4—Cut surface of the liver from a section adjacent to that of figure 3 showing a large blood vessel in relation to the lesions in the parenchyma. One-half normal size.

vessels, parasitic lesions, abscesses, and tumors, were recorded.

Illustrations of telangiectasis are presented in figures 1-4. The lesions in these photographs are spherical to slightly irregular dark areas in the parenchyma of the liver. The size of these lesions varies between those just visible to the unaided eye and those as large as 2 cm. in diameter. In these figures, the distribution and size of the lesions do not follow a pattern in proportion to the diameter of the blood vessels. Lesions of variable size can be seen scattered throughout the parenchyma of the organ. However, in figure 1, some lesions are comparable in size to the uniformly smaller lesions presented in figures 2, 3, and 4. Although telangiectatic lesions may occur throughout the parenchyma of the liver, by far the most common pattern is the presence of locally involved areas. Telangiectasis is not consistently found in any one region of the liver. The incidence of all visible telangiectatic lesions found in a total of 1,570 carefully examined bovine livers is recorded in table 1. These data include the presence of lesions in otherwise normal livers as well as in those showing other pathological lesions. Of the 1,570 livers examined, 61.8 per cent showed telangiectasis. Of the 1,114 livers passing meat inspection, 53.8 per cent were affected

TABLE 1—Incidence of Telangiectasis in the Bovine Liver* with Relationship to the Classes of Animals Encountered at Slaughter

	Normal (passed inspection)		Fluke (condemned)		Abscess (condemned)		Telang (condemned)	Other conditions
	No Telang	Telang	No Telang	Telang	No Telang	Telang		
BULLS 2–6 years	18	31+		5+	4	2+		3 Traumatic No Telang
		7++				1++		
		1+++						
STEERS (not in feed lot)	104	31+				1+		
		10++						
STEERS (in feed lot)	150	203+	18	78+	7	20+		3 Traumatic No Telang
		90++		31++		27++		1 Traumatic Telang +
		25+++		15+++		5+++	1++	
		4++++		8++++		2++++	3++++	
CALVES (not in lot)	10							
CALVES (in feed lot)	63	20+	14	23+	1	2+		
		1++		7++		1++		
				2+++				
HEIFERS	19	8+	2	26+	1			
		4++		11++		1++		
				2+++				
				1++++				
FIRST-CALF HEIFERS	30	17+	3	6+		6+		
		7++						
		2+++		1+++				
							1++++	
COWS 2–6 years (non-bred)	86	70+	16	20+	2	2+		
		16++		12++		2++		
		1+++		9+++			2+++	
				2++++			1++++	
COWS 2–6 years (bred)	32	32+	12	9+	3	6+		
		22++		1++		5++		
						3+++		
							3++++	
Sub-totals		412+		167+		39+		6 Traumatic No Telang
		157++		62++		37++		1 Traumatic Telang +
		29+++		29+++		8+++	3+++	
		4++++		11++++		2++++	8++++	
TOTAL	512	602	65	269	18	86	11	7

* Severity of Telangiectasis lesions: +one to four, ++ four or more local, +++ several involving entire liver, ++++ severely involved.

with varying degrees of telangiectatic lesions. Of the 456 livers condemned for human consumption, 81.8 per cent showed telangiectatic lesions. Livers condemned because of fascioliasis showed 80.6 per cent telangiectasis; those with abscesses, 82.7 per cent; and those involved with traumatic adhesions, 14.3 per cent.

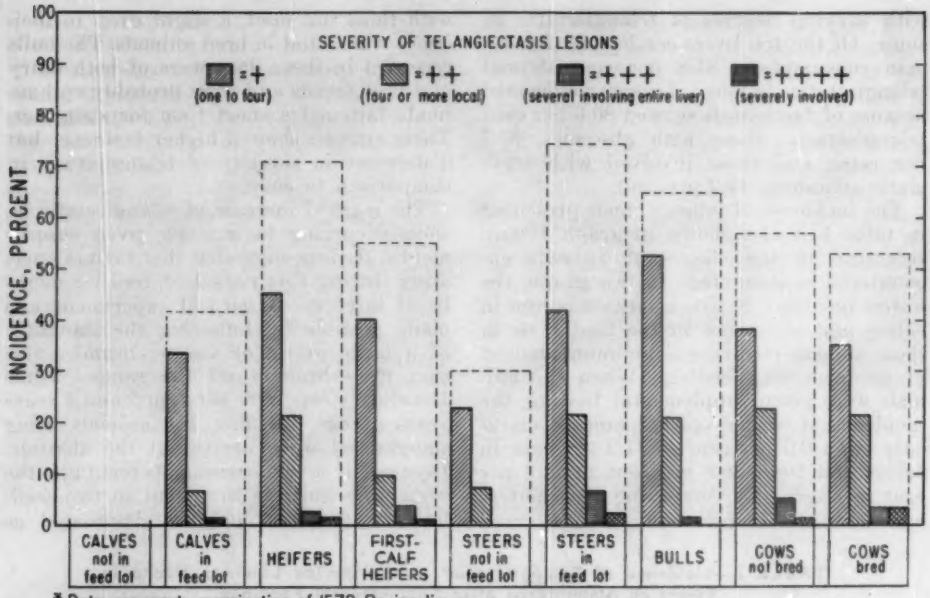
The incidence of telangiectasis presented in table 1 is also shown in graph 1 with reference to the classes of animals encountered at slaughter. In this graph, the lowest incidence of this condition occurs in calves and steers not in the feedlot or in those animals receiving a minimum amount of supplemental feeding. When the animals were given supplemental feeding, the incidence of telangicetasis increased markedly from 0.0 per cent to 41.9 per cent in calves and from 28.8 per cent to 74.3 per cent in steers. By comparing true heifers with first-calf heifers and the cows bred with those not bred, a slight drop in incidence was found in bred animals. The bulls recorded in these data were of both dairy and beef breeds and were probably cull animals fattened a short time for slaughter. These animals showed higher incidence but a decrease in severity of telangiectasis in comparison to cows.

The marked increase of telangiectatic lesions occurring in animals given supplemental feeding suggested that factors operating during this period of feeding might be of interest. A natural experiment was made possible by following the slaughter of a large group of steers (branded $\overline{oo}$) that were brought off the range. These Hereford steers were between 3 and 4 years years of age, the first 146 animals being slaughtered upon arrival at the abattoir. Because of a low dressing percentage, the remaining animals were sent to two feedlots for finishing and are designated as

TABLE 2—Incidence of Telangiectasis* in the Bovine Liver as Found in Steers $\overline{oo}$ Slaughtered after Supplemental Feedings

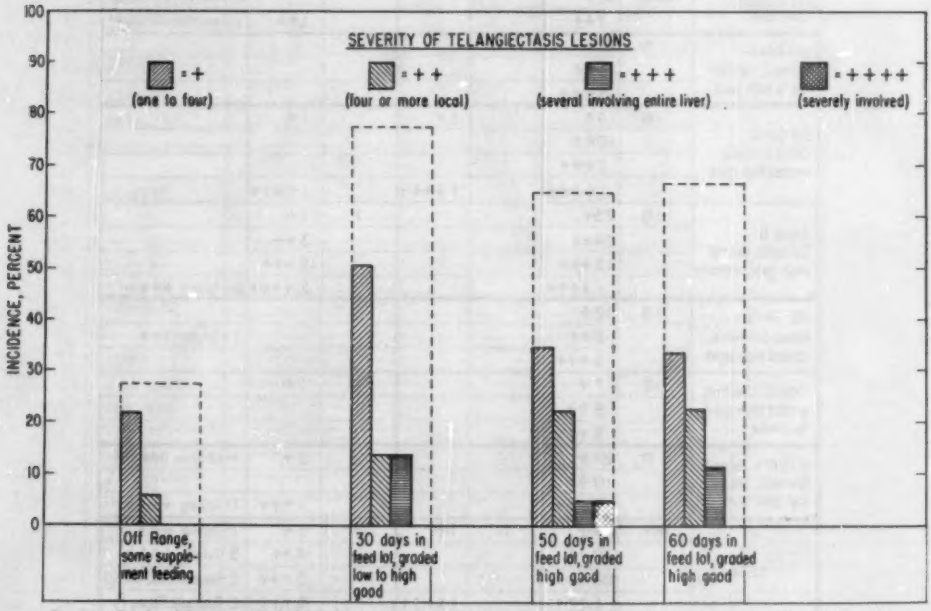
Time in Feed Lot	Normal (passed inspection)		Fluke (condemned)		Abscess (condemned)		Miscellaneous
	No Telang	Telang	No Telang	Telang	No Telang	Telang	
ON RANGE (controls)	104	31+					2-Traumatic (no Telang)
		7++				1++	1-Traumatic (Telang+)
30 DAYS Group I- graded low to high good	5	11+					
		3++					
		3+++					
50 DAYS Group I-Napa, graded high good	16	14+	1	1+		1+	
		10++					
		2+++					
		1++++		1++++		1++++	
Group II Corcoran, graded high good to choice	10	23+			1	1+	
		24++				3++	
		12+++				2+++	
		1++++				2++++	2-Telang ++++
60 DAYS Group I - Napa, graded high good	12	12+					
		8++					1-Telang +++
		3+++					
Group II-Corcoran, graded high good to choice	12	7+			3	1+	
		8++					
		3+++					
80 DAYS Group II-Corcoran, graded high good & choice	8	21+			3	2+	1-Adhesion (Telang+)
		10++					
		5+++				1+++	1-Telang ++++
TOTAL	167	119+	1	1+	7	5+	1-Telang +++
		70++				4++	3-Telang ++++
		28+++				3+++	2-Traumatic (No Telang)
		1++++		1++++		3++++	2-Traumatic (Telang+)

*Severity of Telangiectasis lesions: + one to four, ++ four or more local, +++ several involving entire liver, ++++ severely involved. Data represents examination of 418 Bovine livers.

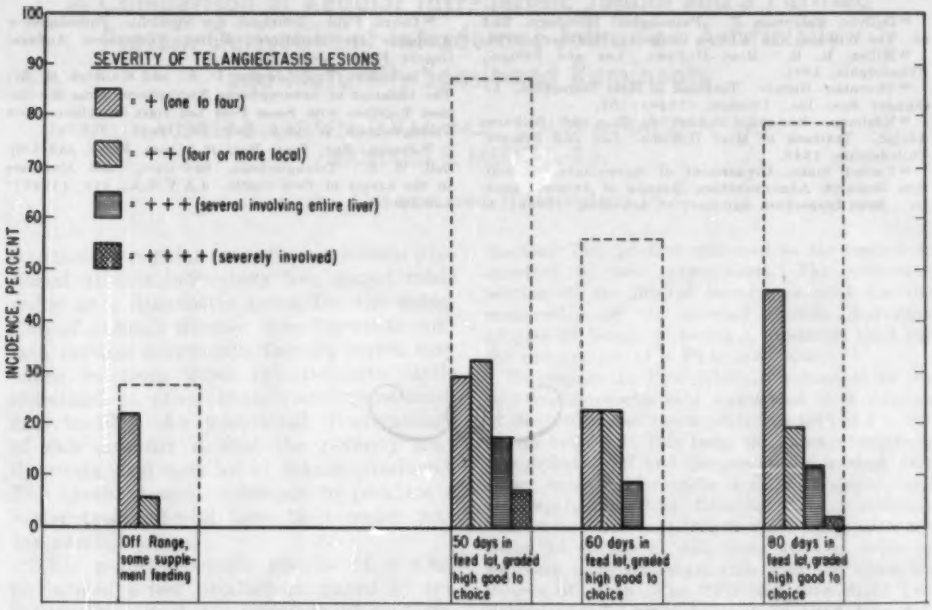


* Data represents examination of 1570 Bovine livers.

Graph 1 — Incidence of telangiectasis* encountered in various classes of cattle at slaughter.



Graph 2—Incidence of telangiectasis encountered in steers oo, group I, slaughtered after supplemental feedings.



Graph 3—Incidence of telangiectasis encountered in steers $\overline{55}$, group II, slaughtered after supplemental feedings.

groups I and II. The data obtained at the first and subsequent slaughters of these animals are presented in table 2.

To visualize these data more clearly, bar graphs of each group are presented by graphs 2 and 3. After thirty days in the feedlot, 22 animals from group I were slaughtered, showing a rise in incidence of telangiectasis from 27.4 per cent to 77.3 per cent (graph 3) with a marked increase in severity of the lesions. This group showed a fluctuating decrease in incidence and severity of the lesions after being held in the feedlot for longer periods.

The essential difference between the steers comprising groups I and II consisted of feeding management and feed quality. Although it was not possible to obtain the details of this variation, the significant fact remains that the incidence and severity of telangiectasis is correlated with feedlot conditions. It is of interest that group I averaged forty-nine days in the feedlot with an average daily gain in weight of 0.83 lb., while group II averaged fifty-six days in the feedlot with an average daily gain of 2.28 lb.

In summary, it can be said that the incidence of telangiectasis is correlated more with feeding conditions than with sex and age variations. Telangiectatic lesions tend to be larger in older animals and in those animals placed in the feedlot for the longest period of time.

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A Comparison of Regular Intradermic Johnin and a Purified Protein Derivative of Intradermic Johnin on Artificially and Naturally Sensitized Ruminants

AUBREY B. LARSEN, D.V.M., M.S.; LOUIS A. BAISDEN, Ph.D.; RICHARD S. MERKAL, B.S.

Auburn, Alabama, and Washington, D. C.

ALTHOUGH regular intradermic johnin produced at this laboratory has considerable value as a diagnostic agent for the detection of Johne's disease, it is known to contain certain nonspecific factors which may cause reactions when injected into cattle sensitized to other than homologous acid-fast bacilli.[1] An additional disadvantage of this product is that the potency may fluctuate with each lot of johnin produced. For these reasons, attempts to produce a better test material have been under way for several years.[2]

This report presents results of a comparison of a test product prepared by trichloroacetic acid precipitation of material from Johne's culture filtrate with regular intradermic johnin. This comparison included both potency and specificity tests on artificially sensitized goats and cattle at the laboratory and potency tests on cattle in the field. Similar materials previously prepared and tested by Baisden *et al.*[2] indicated that they were more specific than standard johnin when tested on artificially sensitized animals. However, the increase in specificity did not reach statistical significance.

PROCEDURE

Both the regular intradermic johnin and the precipitated product, which will be referred to as "johnin PPD," were prepared from cultures of strain 18 *Mycobacterium paratuberculosis* grown on Dorset and Henley[3] synthetic medium. After six weeks' incubation at 38 C., they were removed from the incubator and placed in flowing steam for three hours. The contents of each culture flask were then filtered through filter paper, the bacilli discarded, and the filtrate divided into two portions. One portion was made into regular intradermic johnin by the same procedure as that described by Dorset and Henley[3] for tuberculin production. This product was used as the control or standard in these experiments. The remaining portion of the filtered liquid was used for the preparation of the purified protein derivative (PPD) of johnin following a procedure used for the preparation of a PPD tuberculin.[4]

To prepare the PPD johnin, 1 volume of 40 per cent trichloroacetic acid was added to 9 volumes of the filtrate and the mixture was held at 7 C. for twelve hours. At this time, the supernatant fluid was siphoned off and the precipitate washed with 1 per cent trichloroacetic acid, centrifuged, and the supernatant fluid discarded. The precipitate was washed and centrifuged three times with ten times its volume of anhydrous acetone, followed by three more washings, each with ten times its volume of ether. The PPD was then dried and pulverized. To reconstitute this dried material, the following three solutions were made up for the diluent:

1) M/15 Na_2HPO_4 in distilled water.
2) M/15 KH_2PO_4 in distilled water.
3) 20 parts glycerine, 1 part phenol, and 79 parts distilled water.

These solutions were used in the following proportions for the diluent:

34 per cent solution 1.
16 per cent solution 2.
50 per cent solution 3.

The PPD johnin was dissolved in this diluent at the rate of 2.5 mg./ml. and then tested simultaneously with regular intradermic johnin at this laboratory on two groups of sensitized goats and two groups of sensitized cattle. Each of the four groups consisted of 5 animals. One group of 5 goats and one group of 5 cattle were artificially sensitized by means of a previously described intraperitoneal injection[5] with *M. paratuberculosis* and the second group of each species was sensitized with *Mycobacterium tuberculosis* var. *bovis*.

In conducting these tests, the regular intradermic johnin and the PPD johnin were each injected in 0.2-ml. quantities in four serial twofold dilutions duplicated on each animal. A total of 528 injections was made in this manner. The regular johnin was used as the standard, and the relative potency of the PPD johnin on both homologously and heterologously sensitized animals was the basis for evaluating the specificity. This was accomplished by means of a statistical design described by Wadley[6,7] and used by Baisden *et al.*[2]

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, U.S.D.A., Auburn, Alabama (Larsen and Merkal), and the Animal Disease and Parasite Research Branch, Agricultural Research Service, U.S.D.A., Washington, D. C. (Baisden).

After 25 normal cattle at this laboratory were each injected with 0.2 ml. of the PPD johnin at the same level in the cervical region and no evidence of a reaction was observed in any of them, it was decided that this material would be suitable for field trials on naturally sensitized animals.

The cooperation of the Animal Disease Eradication Branch, Agricultural Research Service, was obtained and simultaneous comparative tests were conducted in the field on 1,134 cattle in 25 herds in which Johne's disease was known to exist. Each animal was injected with 0.2 ml. of regular intradermic johnin on one side of the neck and 0.2 ml. of the PPD johnin at a level of 2.5 mg./ml. in the same area on the opposite side. These sites were observed for evidence of a reaction forty-eight hours later.

RESULTS

In the statistically designed experiment on goats, a significant increase in specificity was observed with the PPD johnin, while in a similar experiment conducted on cattle an increase in specificity was observed which did not reach statistical significance. In a previous experiment not reported in this paper, a similarly prepared PPD johnin was tested against regular johnin and found to be more specific but not of statistical significance. From these three experiments and from previous experiments reported by Baisden *et al.*,[2] the evidence is clear that PPD johnins are sufficiently superior to regular johnin when tested on artificially sensitized animals to warrant accepting them as being more specific, almost to the point of statistical significance.

A total of 95 reactors, to one or both johnins, was disclosed among the 1,134 cattle tested. These cattle were tested by several individuals because of the locations of the herds. The reactors were divided into two groups. Group 1, consisting of 31 animals, was examined by individuals who did not indicate reaction size, but stated only whether the animal was a reactor or suspect, while the 64 reactors in group 2 were given p or x values and classed according to reaction size.

Of the first group of 31, 30 reacted and 1 was negative to PPD. The animal which was negative to PPD was among 17 which were positive reactors to regular johnin. Of the remaining animals, 3 were suspicious and 11 were negative to regular johnin. The individuals conducting these tests stated that the PPD reactions were more pronounced and firmer than those elicited by regular johnin.

The 64 animals in group 2 were all positive to PPD. Sixty-one of these reacted to regular johnin also and 3 were classed as suspicious. The values of the regular johnin reactions and the PPD reactions in relation to pea size (p) were averaged separately and the two averages compared. It was found that the PPD reactions on the 64 reactors averaged 0.5 p larger than the reactions elicited by the regular johnin.

In both groups combined, 77 cattle reacted to both test products (table 1). Since the use of either product would have resulted in the detection of these cattle, it is not necessary to discuss them further.

Three of the 17 animals which did not react to both products to the same degree were not slaughtered; however, suitable intestinal specimens for laboratory examination were obtained at postmortem examination from the remaining 14, and microscopic examination for typical, small acid-fast bacilli was made. Acid-fast bacilli were not found in the material from the 3 animals which were positive to PPD johnin and suspicious to regular johnin, but they were found in material from 3 of the 11 animals that elicited positive reactions to PPD johnin and were negative to regular johnin. Such bacilli were also found in

TABLE 1—Results of Differential Testing of 95 Reactors from 1,134 Cattle Tested with Both Regular and Purified Protein Derivative (PPD) Johnin

Group		Total No.	Comparative No. reactors regular johnin			Comparative No. reactors PPD johnin		
			pos.	susp.	neg.	pos.	susp.	neg.
PPD johnin	Positive	94	77	6	11	....	....	....
	Suspicious	0	0	0	0	....	....	....
	Negative	1	1	0	0	....	....	....
Regular johnin	Positive	78	....	....	....	77	0	1
	Suspicious	6	....	....	....	6	0	0
	Negative	11	....	....	....	11	0	0

the 1 animal that reacted to regular johnin but failed to react to PPD johnin.

Discussion

The results indicate that PPD johnin at the level used was more potent than regular johnin. The increase in reaction size caused by the PPD johnin resulted in 17 additional animals, among the 1,134 tested, being classed as reactors. It should be noted that 3 of these were found to contain typical small acid-fast bacilli in the intestinal tract and would have continued to be a source of infection had they remained in the herd. The PPD johnin also showed an increase in specificity over regular johnin in laboratory tests on artificially sensitized goats and cattle.

Since exact weights of this powdered material can be dissolved in the diluents, it should be possible to produce a johnin more nearly standard in its potency from one lot to another than can be obtained by the regular procedure for producing intradermic johnin. Regular johnins vary so much that occasional lots must be discarded as unsatisfactory. Although it is recognized that equal weights of PPD powders may not contain exactly equal quantities of active material, it is believed that there is a greater chance of controlling potency from lot to lot of the PPD's than would be the case with regular johnins. It may be possible to store the PPD johnin as a powder for long periods without loss of potency since this has been found true in the case of PPD tuberculin. These studies indicate that the purified protein derivative of johnin has several advantages over regular johnin for routine intradermic testing, and PPD johnin warrants continued investigation.

Summary

A purified protein derivative (PPD) of johnin, prepared by trichloroacetic acid-precipitation of Johne's bacilli culture filtrate, appears to be more specific than regular johnin when tested on artificially sensitized goats and cattle. This product was put in solution at 2.5 mg./ml. and injected intradermically in 0.2-ml. quantities in each of 1,134 cattle in herds infected with Johne's disease along with equal quantities of regular johnin on the opposite side of the neck.

Both products gave negative reactions on 1,039 animals. Reactions classed as positive were elicited in 95 animals; 94 of these reacted to the precipitated product. Regular johnin elicited 78 reactions classed as positive, 6 classed as suspicious, and 11 classed as negative in these 95 animals. One animal reacted to regular johnin but not to the precipitated product.

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The Teeth of the Ox in Clinical Diagnosis
IV. Dental Fluorosis

NORMAN L. GARLICK, D.V.M.

Tacoma, Washington

Parts I, II, and III of this series, which appeared in past issues of the American Journal of Veterinary Research, *gave the essential histology, embryology, physiology and gross anatomy of the teeth of cattle, as well as the observed developmental anomalies, injuries, and general pathology of the teeth. Included with Part I was a bibliography for suggested further study.*

Dental Fluorosis

Dental fluorosis occurs as a result of continued ingestion of excessive amounts of fluorides and in the ox has been observed to affect only the developing permanent dentition. Apparently, the placental membranes are able to screen out fluorides (fig. 1 and 2). Permanent teeth, if fully formed and erupted before ingestion of excessive amounts of fluorides begins, show no lesions nor deleterious effects (fig. 3).

Fluorine is a common element which, being chemically very active, always occurs in nature in combination with other elements. Fluoride salts are found in varying amounts in animals, soils, vegetation, and water. All herbivorous animals continuously ingest and excrete moderate amounts of it under normal conditions. In some wells, the water contains concentrations of fluorides sufficiently high to cause changes in developing permanent teeth. Other sources of fluorides are depositions on the surface of ingested vegetation (either from industrial effluents, volcanic eruptions, dusts, soils, or continued use of fluoride-bearing insecticides), the feeding of mineral supplements which have as a base inadequately defluorinated rock phosphate, the feeding of fluoride-bearing brewers by-products, and perhaps others. Endemic fluorosis areas are limited geographically. Regardless of the source, the effects on developing permanent teeth are the same. Lesions vary with the changing concentrations in the blood stream, the age at time of ingestion, and duration of ingestion.

Up to a point, the excretion of fluorides in the urine can nearly equal the excess amounts being absorbed into the blood stream via the intestine. As ingested amounts increase, the percentage of fluorides retained by the body increases, the fluorides accumulating within the skeletal tissues. When ingestion of excess fluorides ceases, excess excretion will continue for a variable period of time until a normal concentration is reached in the blood stream. Because of the lag between discontinued ingestion and eventual normal concentrations, there is a comparable lag also before dental changes cease to occur. Urinalyses have proved to be of value in that they reflect, within limits, the blood concentrations of fluorides.

Because the developing enamel is sensitive to excess concentrations of fluorides, the enamel changes serve as a constant index, reflecting concentrations over normal. The period of ingestion of excess fluorides by young animals with incomplete permanent dentition can be closely approximated. The changes are permanent.

Excess fluorides have three effects on developing enamel—mottling, disturbed calcification, and hypoplasia.

Mottling of Enamel.—The Dorland[1] definition of mottling, "a condition of a tissue in which it is spotted with patches of color," will be used. (Other workers, however, refer to mottling only as a variation in the opacity of the enamel, adding another factor, that of pigmentation, discoloration, or stain.)

Mottling of the enamel will be the first, and if ingestion levels are relatively low, will be the only manifestation of increased fluoride ingestion. It is not deleterious to the health of the animal and will be produced by concentrations of fluorides well below those necessary to produce observable systemic disturbances. Therefore, mottled teeth serve as an index of fluoride in-

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The author is engaged in veterinary consultation and research work in Tacoma.

gestion during the formative period of the permanent dentition. Its absence in permanent teeth which were formed during a period of suspected ingestion of excess fluorides positively eliminates fluorides from clinical consideration. Allowance must be made for the time lag between eruption and discoloration as will be explained later.

The Appearance of Mottled Enamel.—The pigmentation in mottled enamel varies in color from a very light yellow through all shades of brown to an ebony black. Greens are also seen. While the darker colors are usually seen on higher levels of ingestion, some of the darkest colors have been observed in animals on relatively low levels. Since the pigmentation varies in intensity in various portions of the tooth, blending in gradually with surrounding lighter areas, it serves to differentiate this condition from that of enamel flecks already described in Part III. Mottling takes a horizontal pattern, following the pattern of enamel maturation, though in the presence of enamel fissures it may follow the defect vertically due to acceleration of pigmentation along the defect. Pigmentation shows up first on those areas from which the enamel cuticle tends to wear away the earliest.

When an affected tooth first erupts, the mottling is not yet in evidence (fig. 4). A variable period of time is necessary before the pigmentation is apparent. The color

Fig. 1—Normal temporary incisor teeth of a calf 4 months and 8 days of age. The dam had been ingesting toxic quantities of fluorides almost continuously during the four years prior to the birth of this calf.

gradually deepens as time goes on but has a tendency to fade in old age. Since the newly erupted teeth may normally present a slightly yellow surface, due to staining of the tissues overlying the enamel prisms,

Fig. 2—Emaciated cows suffering from systemic and dental fluorosis. The normal 1-day-old calf shows neither systemic nor dental fluorosis. The black cow is standing cross legged to relieve the pressure on the medial forelimb digits, and the cow being milked has adopted a stance to relieve pain in the front feet. The bruised areas on the latter's knees are from crawling. All 3 are eating hay in preference to a good stand of contaminated pasture grass.

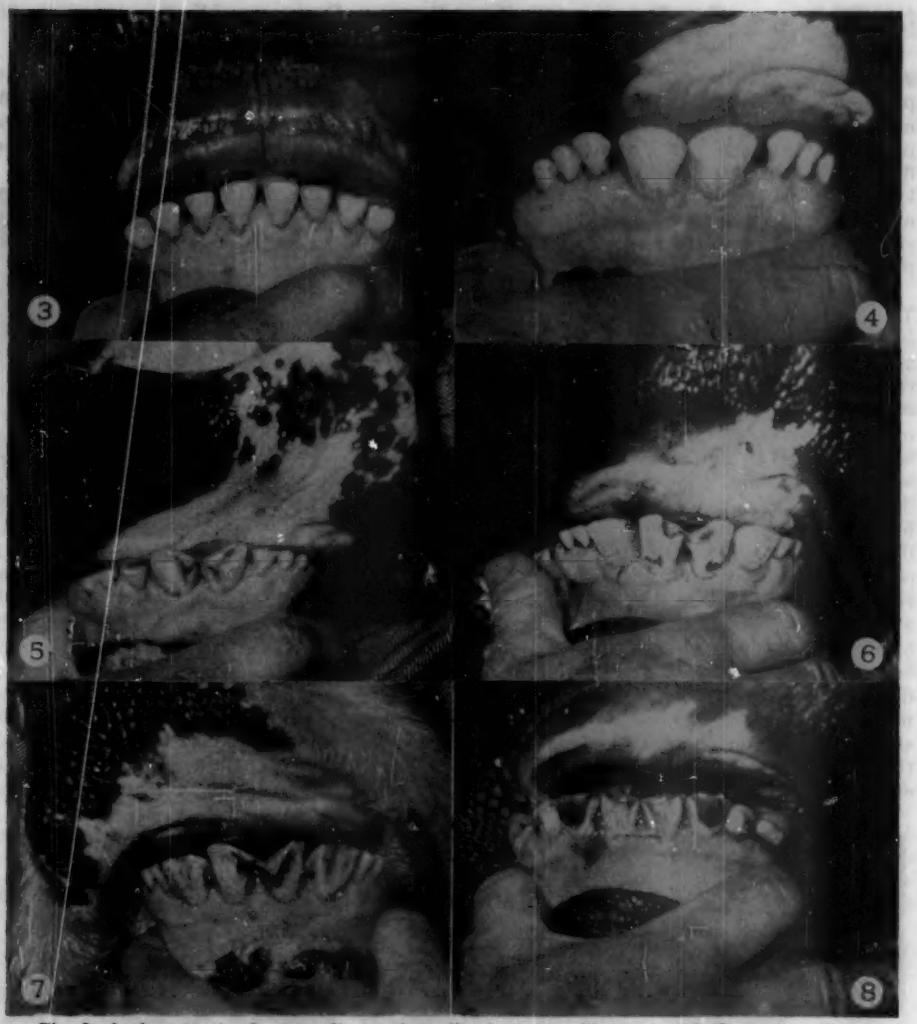

Fig. 3—Incisor teeth of unusually good quality in a cow 13 years and 10 months of age which had ingested toxic quantities of fluorides for a total of six and a half years following completion of dental formation.

Fig. 4—Incisor teeth of a heifer 2 years and 1 month of age. The permanent pair appear normal, but later examinations revealed a considerable degree of damage by fluorides.

Fig. 5—Dental fluorosis in the permanent incisor teeth of a heifer 2 years and 8 months of age, showing accelerated attrition and mottling. The pigmentation is dark brown. The temporary teeth are normal.

Fig. 6—Dental fluorosis in the permanent incisor teeth of a heifer 3 years and 1 month of age, showing mottling, disturbed calcification and hypoplasia. The affected teeth are light yellow-brown with shadings to dark brown. Hypoplasia can be seen near the gingival margins of the central incisors, the pattern being roughly horizontal.

Fig. 7—Dental fluorosis in the permanent incisor teeth of a heifer 3 years and 3 weeks of age, showing mottling and accelerated attrition. (Compare with heifer, fig. 6, from the same herd and only one week older.)

Fig. 8—Dental fluorosis in the permanent incisor teeth of a cow 3 years and 7 months of age. The fourth incisor teeth, just erupting, show no discoloration, although they are chalky and without luster. The second pair shows two vertical fissures outlined by deep pigmentation which follows the defects.

the teeth should not be "read" too soon. Mottled enamel is almost always bilateral (fig. 5-9), although when the duration of fluoride ingestion has been short and the development of an individual tooth has lagged, the mottling may occur on only one.

The nature of the pigment in mottled enamel is unknown. It has been postulated by Black [2] that a potential pigment is deposited within the enamel at the time of formation of the tooth if excess fluorides are being ingested. He called this potential pigment "pre-brownin." The "pre-brownin" is said to react with some environmental influence to produce the visible pigmentation which he called "brownin." Other theories indicate that the color may be due to extraneous pigments which invade the defective enamel. The intensity of pigmentation is almost as great on the last cheek teeth, if affected, as it is on the incisor teeth, tending to refute the theory that the pigment is activated by light. The

Fig. 9—Dental fluorosis of the permanent incisor teeth of a cow 3 years and 6 months of age. Mottling and disturbed calcification are progressively more evident from the central pair outward, although the fourth incisors have not been erupted long enough to show much discoloration.

Fig. 10—Dental fluorosis in the permanent incisor teeth of a heifer 2 years and 5 months of age. Although softening of these teeth is evident, a light yellow mottling is just beginning to show.

Fig. 11—Dental fluorosis in a cow 5 years and 2 months of age, which ingested toxic quantities of fluorides only after the age of 1 year and 9 months. The first pair of teeth is normal, the second pair is mottled with light brown pigment, but with no sign of accelerated attrition, while the third and fourth pairs show extensive mottling and disturbed calcification.

Fig. 12—Dental fluorosis in a cow 10 years and 2 months of age, the result of ingesting toxic quantities of fluorides after 2 years of age. The first two pairs of incisors are of excellent quality, while the third and fourth pairs have only the roots persisting. The rapidity of wear of the fourth pair is indicated by the deeply stained poor quality secondary dentine in the center.

pigments can not be removed without removing the substance of the enamel itself.

Disturbed Calcification.—Two thirds of the inorganic constituents of enamel is deposited during the maturation period. In the ox this takes about six months, in the case of the incisor teeth, ending at eruption or shortly thereafter. When fluorides are being ingested at a level above that which produces only mottling, the result will be both mottling and softening of the enamel. The softening is probably due to incomplete calcification and to deposition of inorganic salts of inferior quality compared to the normal. If only a portion of any pair of teeth is involved, the mottling and softening will occur in the same pattern on both. If the softening is extensive, the teeth will wear away faster than normal.

Fig. 13—Dental fluorosis of an incisor tooth with extensive mottling and extreme disturbance of calcification. Where the hornified layer of the enamel cuticle has peeled off, the enamel has crumbled like chalk.

(The use of the term "corrode" found in human dental literature must be explained. Usually the term indicates a chemical reaction on the surface of the material involved. McKay introduced the term and dental pathologists use it to describe the mechanical accelerated wearing away of teeth softened by fluorides. No chemical corrosive action is implied. It was an unfortunate choice. In the opinion of the author, increased retrogression, or accelerated attrition could be used to better advantage.)

If fluoride ingestion has been excessive and relatively constant during the entire period of permanent dentition formation, a pattern of wear will develop. Since it has a cumulative effect, the first teeth to form will be of better quality than those which develop later. This is the reverse of the usual variation in quality.

At the time of eruption, fluoride-softened teeth present in lusterless, opaque-white enamel, and a differentiation from the condition previously described as "chalky teeth" is not possible, visually. Only after the mottling shows up can a positive differentiation be made. If non-removable discoloration does not show up in six months or so, the chalky condition is not due to fluoride ingestion.

Moderate softening of the teeth by fluorides may not be grossly evident for some time, but if the rate of attrition is faster than normal and mottling appears, the enamel has probably been softened by fluorides (fig. 10).

Patterns of Accelerated Attrition of Fluoride-Softened Teeth.—If intake of excess fluoride has not been constant, only those teeth which had reached the enamel calcification period at the time of that excessive ingestion will be affected (fig. 11 and 12). Thus, accelerated attrition patterns can effectively demonstrate the time at which excessive fluoride ingestion took place in an animal up to the age of 4 years. Since the quality of the dentine is not noticeably varied from the normal, it will resist attrition better than the affected enamel. Since affected teeth wear at an accelerated rate, the deposition of secondary dentine is accelerated. If the attrition is

Fig. 14—Dental fluorosis of a cow 7 years and 9 months of age, which ingested toxic quantities of fluorides from contaminated pasture grasses during the first 2 years and 6 months of her life. All of the teeth are affected but in a decreasing degree as age advanced. The fourth pair, which probably erupted about one year after ingestion of excess fluorides ceased, shows light brown mottling and demonstrates the tapering-off period.

such that the pulpal portions of the teeth are closely approached, they are apparently more sensitive than normal. The hornified layer of the enamel cuticle in fluoride-softened teeth is more highly developed than normal and serves to delay the attrition of the affected enamel beneath (fig. 13). When extraction forceps are applied to badly affected teeth, the cuticle is broken and the underlying enamel will crumble readily. Darkly pigmented diffuse or pinpoint areas of erosion may appear on the labial surface.

Accelerated Attrition of Fluoride-Softened Incisor Teeth.—The rate of wear of affected incisor teeth depends upon the degree of softness. The enamel at the incisal margin is soon lost and the medial and lateral margins wear away, giving the tooth a narrower appearance. The central dentinal portion remains the highest, the tendency being to round off at the edges of the occlusal surface (fig. 14). The teeth may continue to wear until they are level with the gingival margin, the roots persisting in the alveoli as useless pegs. Affected teeth do not fall out (fig. 12). If the excessive intake has been constant during the four-year formative period, the enamel is progressively softer from the first to the fourth pairs of incisors.

Accelerated Attrition of Fluoride-Softened Cheek Teeth.—If an animal's incisor teeth do not show the effects of fluoride ingestion, neither will the cheek teeth, but when fluoride lesions are present on incisor teeth they will also be on the cheek teeth and at the same point in the chronological developmental stage. In the living subject, examination difficulties interfere with the identification of lesions on the cheek teeth. However, patterns of wear can be recognized and can be differentiated from normal attrition. If the lesions are present on a certain pair of incisor teeth, examination of the cheek teeth which were developing at the same time will reveal similar defects.

If the ingestion of excessive amounts of fluorides has been constant during the dental-formative period (as from drinking water) the first cheek teeth to form, because of the accumulative effect, will be of better quality than the rest and will resist attrition the longest, while the third permanent maxillary premolar will be the softest tooth. The first mandibular molar will be quite high on the masticatory table where it opposes the third maxillary premolor (fig. 15). The first maxillary molar will be softer than its mandibular counterpart and will wear in favor of the latter where they are opposed. However, the first maxillary molar, being harder than the second mandibular molar, will be long on its posterior portion. The second mandibular molar, where it opposes the second maxillary molar, will be high (fig. 16). Variations in softness will be less prominent in the third molar teeth. The permanent premolar teeth, erupting after the molars, will be the last to show accelerated attrition. Because the premolar teeth have less erup-

Fig. 15—Dental fluorosis of the mandibular cheek teeth of a 4-year-old steer. Disturbed calcification is indicated by the high fourth and low fifth cheek teeth.

Fig. 16—Dental fluorosis of the permanent cheek teeth of a 4-year-old steer, showing mottling, hypoplasia, and the pattern of accelerated attrition. (The fact that the first premolar has failed to form has no relationship to the lesions of dental fluorosis.)

Fig. 17—Dental fluorosis of an incisor tooth, showing extreme hypoplasia at the tip which follows an irregular horizontal pattern. The mottling, which is deep brown to black, outlines the area denuded of enamel cuticle.

Fig. 18—Dental fluorosis of an incisor tooth. The diffuse pits and two horizontal grooves, in the lower third of the crown, indicate hypoplasia. Disturbed calcification is very evident. The mottling in the pits is emphasized by accumulated forage pigments.

Fig. 19—Dental fluorosis of the incisor teeth of a 3-year-old steer from an area in Texas where the drinking water is high in fluorides. The mottling, accelerated attrition, and hypoplasia are extreme.

tion potential than the longer molar teeth, the attrition-caused variations are less evident. (Since a normal attrition pattern demonstrates a *low* first mandibular molar, and the pattern in fluoride-softened teeth results in a *high* first mandibular molar, a careful examination will readily differentiate the two conditions in older animals.)

Clinically, the extent of accelerated attrition is the most important factor, the useful life of the affected teeth being shortened. If the projecting portions of the affected teeth reach the gingiva of the opposing dental arch, traumatic gingivitis is produced. Alteration of the masticatory table is earlier than normal so that satisfactory comminution of food is impossible at a relatively earlier age.

Hypoplasia of Enamel.—If ingestion levels of fluorides are relatively high during the appositional period of amelogenesis, hypoplastic lesions may be in evidence. The lesions may be either horizontal grooves, which follow the incremental pattern, or diffuse pits (fig. 17 and 18). No other form has been observed. Mottling and softening will also be in evidence if the defect is due to fluoride ingestion. The greater amount of hypoplasia will develop if fluoride ingestion begins at the time of birth and is continuous during the first year of life. Those given concentrations of fluoride in the drinking water are more likely to produce enamel hypoplasia than similar concentrations from sources which may not be consumed for six months or so after birth (fig. 19). Hypoplasia appears bilaterally, affecting the permanent dentition only.

SUMMARY

Dental fluorosis is a widespread clinical phenomenon affecting the developing permanent dentition of the ox. It is caused by the ingestion of quantities of fluorides from natural or incidental sources which exceed the average normal daily intake. Pathological conditions which may be in evidence, subject to the limitations covered in the text, are mottling, defective calcification, and hypoplasia of the enamel. Patterns of accelerated attrition are important in determining the relative loss of effective use and length of service of the teeth. Knowledge of the lesions of dental fluorosis is essential in correlating a clinical diagnosis of systemic fluorosis.

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Histopathological Findings in Cows Naturally Infected with *Leptospira Pomona*

W. J. HADLOW, D.V.M., and HERBERT G. STOENNER, D.V.M.

Hamilton, Montana

THE EXPANDING literature on leptospirosis contains few and often only incidental reports concerning the histopathological aspects of the disease in cattle. Only the more informative contributions will be mentioned.

In 1941, Awrorow [1] presented a detailed account of the pathological changes in 40 animals which died during an epizootic of leptospirosis in the Caucasus. He found centrolobular necrobiosis and interlobular round cell infiltration of the liver as well as degenerative changes in the kidneys and myocardium in the acute and subacute cases. Leptospiras were demonstrated in histological sections of liver, kidney, lung, heart, and various lymph nodes—especially the mesenteric lymph nodes.

The lesions in 4 experimentally infected calves were similar to those in the 40 spontaneous cases. Serologically, the isolated strains proved to be closely related to *Leptospira grippotyphosa* although not identical with this type.[11] Jungherr,[5] in Connecticut, described the necropsy findings in 3 cows which died during the acute phase of the disease. Extensive hepatic cell necrobiosis with hemorrhage was found in the liver of 1 cow. The kidneys of another cow showed large areas of tubular necrosis and lymphoid interstitial infiltration. Foci of tubular hypertrophy were found in the kidneys of the third cow. Hemosiderin was present in the spleens. Leptospiras were demonstrated in the liver, the kidneys, and a mesenteric lymph node, but none was isolated from the tissues. Mathews [7] studied the pathological changes in the acute and chronic forms of the natural disease in Texas. The most constant microscopic finding in the liver of the acute fatal form was focal necrosis, usually around the central vein. Extensive albuminous degeneration, necrosis, and pigmentation of the renal tubular epithelium were prominent changes. The spleen was congested and showed marked hemosiderosis while mucosal hemorrhage, necrosis, and ulcer formation were constant findings in the abomasum.

The chronic form was characterized by advanced renal tubular damage with cast formation and interstitial nephritis with beginning fibrosis. The spleens showed marked hemosiderosis. Leptospiras were found in some sections of liver and kidney, but none was isolated from the tissues. Cordy and Jasper [6] studied 16 cattle which died of an acute hemolytic anemia in California. Centrolobular hepatic necrosis and interlobular mononuclear cell infiltration, renal degenerative and interstitial inflammatory changes, and some splenic hemosiderosis were the most prominent microscopic findings. Leptospiras were demonstrated in thin sections of kidney and/or liver of 15 animals and were presumed to be the cause of the disease.

Baker and Little [2] found patchy interstitial nephritis in a group of cows and calves experimentally infected with an organism later identified as *Leptospira pomona*.[4] The most comprehensive account of the histopathological changes in experimentally infected calves was reported by Ungar and Bernkopf [10] following recognition of bovine leptospirosis in Israel. These authors grouped their material according to the three distinct clinical pictures which they recognized. In group 1, the acute fulminant form, which was accompanied by jaundice, and in group 2, the protracted form unaccompanied by jaundice, hepatic cord dissociation and cholangitis were observed. Congestion and hemosiderosis of the spleen were found in these groups. Regressive tubular changes and interstitial infiltrations were present in the kidneys of these two groups as well as in 1 calf (group 3) which recovered after a mild clinical course of the disease. Splenic hemosiderosis was not a feature of the latter group. Leptospiras were demonstrated in the liver and kidneys of calves in the first two groups but only 1 calf of the third group (4 animals) showed leptospiras in the kidneys. The Israel bovine strain of Leptospira, used in this study, has been shown to have an antigenic pattern resembling *L. grippotyphosa*.[11] Reinhard [10] found focal interstitial nephritis in 6 calves which were experimentally infected with a Leptospira subsequently identified as *L. pomona*.[4] Splenic hemosiderosis and focal hepatic necrosis were noted in 1 calf which was examined at an early stage of the disease.

The purpose of this paper is to report

From the U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory (Hadlow), Hamilton, Mont.

Dr. Stoenner is from the Communicable Disease Center, Atlanta, Ga.

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the histopathological findings in several organs of 15 Hereford cows from a herd in which abortion was the most apparent manifestation of spontaneous *L. pomona* infection. The specific clinical diagnosis was established on the basis of the individual serological findings and the isolation of *L. pomona* from 1 cow. In general, the lesions found were consistent with those described for the chronic phase of bovine leptospirosis.

HERD HISTORY

The herd, located in Washington, consisted of 135 females bred to calve in February and March, 1953. In addition, the herd included 34 steers and open heifers. They were wintered in small pastures and had access to a well-constructed cattle barn. The main ration during the winter months consisted of a mixture of good quality native grass and clover hay fed from mangers in a loafing barn. Water from a deep well was provided in concrete water tanks.

The extremely mild winter of 1952-1953 probably favored the rapid spread of leptospirosis through the herd. Because of poorly drained lots and excessive rainfall, the barnyard was muddy throughout the winter. Although the cattle were watered from tanks, they frequently drank from pools in the barnyard and pasture.

All of the cows and heifers had been vaccinated as calves against brucellosis and the herd had been free of this disease since Nov. 16, 1949. The last blood test, Mar. 4, 1953, revealed 6 suspects in 135 animals. The suspects were 2-year-old cows which had been officially vaccinated with strain 19 *Brucella abortus* vaccine. The herd was free of tuberculosis and had been essentially free of abortions for several years preceding the outbreak of leptospirosis. The first abortions occurred on Dec. 24, 1952, with 12 more aborting by Jan. 22, 1953, and an additional 24 by March 4. Most of the abortions occurred during the last trimester of pregnancy, although a few took place as early as the third month. Nearly all of the cows which aborted firmly retained the fetal membranes. A characteristic feature of the aborted fetus was an edematous umbilical cord which characteristically severed close to the abdominal wall. Two of the aborting cows died, presumably from metritis. After March 4, 2 more aborted but the remainder gave birth to normal living calves.

Table 1 shows the serological findings and dates of abortion of the cows from which tissues were obtained for microscopic study. The entire herd was tested on Mar. 4, 1953, at which time the capillary tube test [12] revealed the following titers for *L. pomona:* Negative—26; 1:40—18; 1:160—35; and 1:640 or greater—89. On Apr. 1, 1953, urine specimens were collected from 5 reactors which included 2 cows (390 and 391) whose tissues were studied. *Leptospira pomona* was recovered by guinea pig inoculation from 1 of the 3 reactors not included in this report.

For economic reasons, the owner was forced to reduce the size of the herd and the cows which had aborted were not retained. Tissues from 15 of these cows, which were sent to slaughter through a state leptospirosis indemnity program, form the basis for this study.

MATERIALS AND METHODS

The 15 cows were slaughtered under the supervision of the Federal Meat Inspection Service. The tissues, removed during the postmortem in-

TABLE 1—Summary of the Serological and Histopathological Findings in Cows Naturally Infected with Leptospira Pomona

Cow† (No.)	Serology (*L. pomona*)			Kidneys		Spleen	Liver
	Tested 1-22-53 cap.-tube	Tested 3-4-53		Interstitial infiltrate	Leptospiras	Hemosiderosis	Portal infiltrate
		cap.-tube	aggl.-lysis				
375**	Not tested	1:160	1:500,000	Extensive	—	Moderate	Minimal
376**	Not tested	1:640 or >	1:500,000	Extensive	—	Moderate	None
377**	Not tested	1:640	Not tested	Moderate	+	Marked	Minimal; increased portal connective tissue; nectrotic foci
378**	Not tested	1:640	1:500,000	Moderate	—	Slight	Moderate; increased portal connective tissue
381*	1:160	1:40	1:5,000	Extensive	+	Moderate	Moderate; destruction of several bile ducts
382**	Not tested	1:640	Not tested	Minimal	—	Marked	Minimal
383**	Not tested	1:160	1:500,000	Moderate	+	Moderate	Minimal
384**	Not tested	1:640 or >	1:500,000	Slight	+	Moderate	Minimal
385*	1:640	1:640	1:500,000	Extensive	+	Marked	Minimal
386*	1:640 or >	1:640	1:500,000	Slight	—	Moderate	None; interlobular fibrosis
387*	1:640 or >	1:640	1:500,000	Minimal	—	Slight	Minimal
389*	1:640	1:640 or >	1:500,000	Slight	—	Moderate	None
390**	Not tested	1:640 or >	1:500,000	Moderate	+	Moderate	Minimal; mild fatty metamorphosis
391*	1:640 or >	1:640	1:500,000	Moderate	—	Moderate	None
392**	Not tested	1:160	1:500,000	Slight	—	Marked	None

† Slaughtered 4-15-53; * aborted during period 12-24-52 to 1-22-53; ** aborted during period 1-22-53 to 3-4-53.

spection, were fixed in neutral buffered 10 per cent formalin and then forwarded to the laboratory. Representative blocks of tissue were embedded in paraffin, sectioned at 6 μ, and stained with alum hematoxylin and eosin. Sections, 5 μ thick, were impregnated with silver according to the method of Parã[9] for demonstration of leptospiras. Hemosiderin was demonstrated by Perls' acid ferrocyanide method. Certain selected sections were stained with Crossman's triple stain, Lillie's buffered azure-eosinate stain, Kinyoun's acid-fast stain, Lillie's allochrome stain,[6] and the periodic acid-Schiff stain.

GROSS OBSERVATIONS

Gross observations were limited to the larger portions (3-4 lobes) of the formalin-fixed kidneys. The usual finding was a faint mottling of the cortex with indistinct grayish white foci. An occasional single focus measured up to 6 mm. in diameter. The larger foci frequently were slightly elevated above the subcapsular cortex. A distinctly roughened cortical surface was seen in one specimen and one kidney presented a smoothly nodular subcapsular cortex. The capsules were nonadherent and there was no detectable fibrosis. Usually the cortex was pale. Two kidneys appeared normal except for an occasional grayish 1 to 2 mm. distinct focus deep in the cortex. Only one kidney specimen was without detectable foci. A single kidney was pale and mottled with bright red, small, irregular-shaped hemorrhages throughout the cortex. The medullas presented no gross alterations. The calyces of one specimen contained considerable yellow sandy material and several yellowish brown calculi 4 mm. in diameter.

MICROSCOPIC FINDINGS

Kidneys.—The constant and predominant microscopic finding in the kidneys was widespread focal mononuclear cell infiltration of the interstitial tissues of the cortex and corticomedullary junction (fig. 1). Less frequently, small foci were found deep in the medulla. The areas of exudate varied from large irregular-shaped dense cellular patches to small nodular or linear masses which were periglomerular, perivascular, or intertubular in distribution. The inflammatory infiltration was recorded as extensive (++++) in four, moderate (+++) in five, slight (++) in four and minimal (+) in two, according to the relative degree of involvement. The areas of infiltrate extended from the capsule to the arcuate vessels, where the adventitia of the arteries often was involved. Focal infiltrations were present also in the stroma underlying the epithelium of the minor calyces in several cases. In one kidney, small areas of hemorrhage were found in the interstitial exudate of the cortex.

The interstitial inflammatory cells consisted of small and large lymphocytes, histiocytes and, less frequently, polymorphonuclear neutrophils and eosinophils. Occasionally, plasma cells were conspicuous. The deeply basophilic small lymphocyte was the predominant cell in the smaller foci, while the more extensive cellular patches usually consisted of the less basophilic large lymphocyte and histiocytes. Cells thought to be isolated degenerating tubular epithelial cells were seen in some of the areas of infiltrate. Aggregates of fused cells whose morphology often was

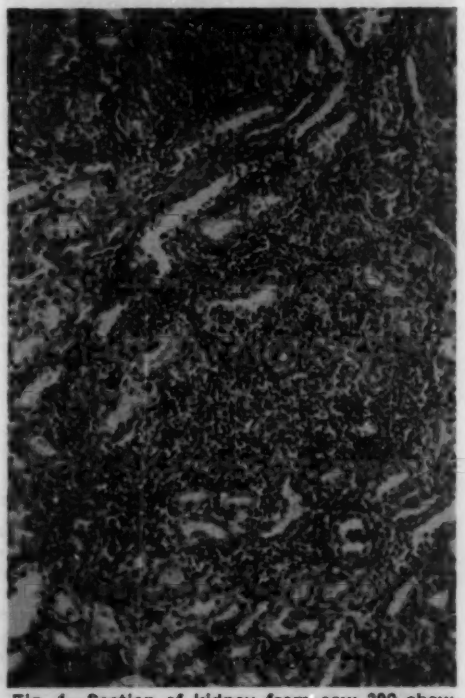

Fig. 1—Section of kidney from cow 390 showing area of lymphocytic interstitial infiltrate in the cortex. Hematoxylin and eosin. x 104.

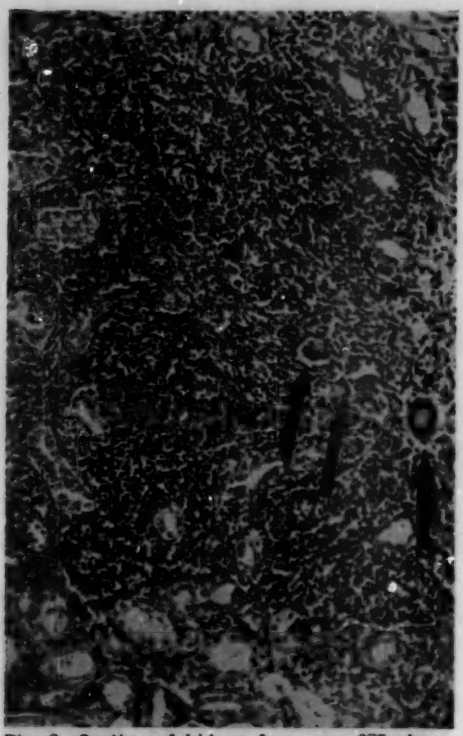

Fig. 2—Section of kidney from cow 375 showing large subcapsular focus of interstitial infiltrate with several giant cells (arrows) and proliferated remnants of the entrapped tubules. Hematoxylin and eosin. x 104.

strikingly suggestive of granulomata formed a conspicuous part of the tissue response in many of the larger interstitial foci (fig. 2). There were numerous multinucleated giant cell formations which resembled those of the Langhans' type (fig. 3). Focal masses of faintly eosinophilic cells formed syncytial aggregates which at times closely resembled cellular remnants of tubules partially disrupted by the interstitial exudate (fig. 4). Often the aggregates of fused cells and the giant cells formed around the entrapped tubules in a manner suggesting their origin from the tubular epithelium (fig. 5). In sections stained by the allochrome procedure, it was found that in reality many of the syncytia as well as the giant cells were of renal tubular cell origin. The tubular basement membrane was found to have disappeared at the site where the cells of the tubules proliferated into the contiguous interstitial exudate as epithelial syncytia (fig. 6). In some instances, a barely detectable (allochrome stain) remnant of the tubular basement membrane circumscribed the bizarre masses of epithelial cells. However, occasionally the syncytia and giant cells were in close apposition with tubules whose basement membranes were thin but without demonstrable defects (fig. 7). Some of these lesions were interpreted as probably representing genuine granuloma formation around dead or damaged tubules. It should be emphasized, however, that in routine hematoxylin and eosin preparations it was not possible to distinguish between the latter lesion and the bizarre epithelial syncytia resulting from apparently abortive attempts to repair the damaged tubules in the absence of intact basement membranes (*see* following).

Proliferation of the tubular epithelium within the areas of interstitial exudate was a conspicuous feature of the microscopic picture in many of the kidneys. The cytoplasm of the involved cells tended to be slightly more basophilic than the normal tubular cells and the nuclei were large and vesicular. At least one prominent nucle-

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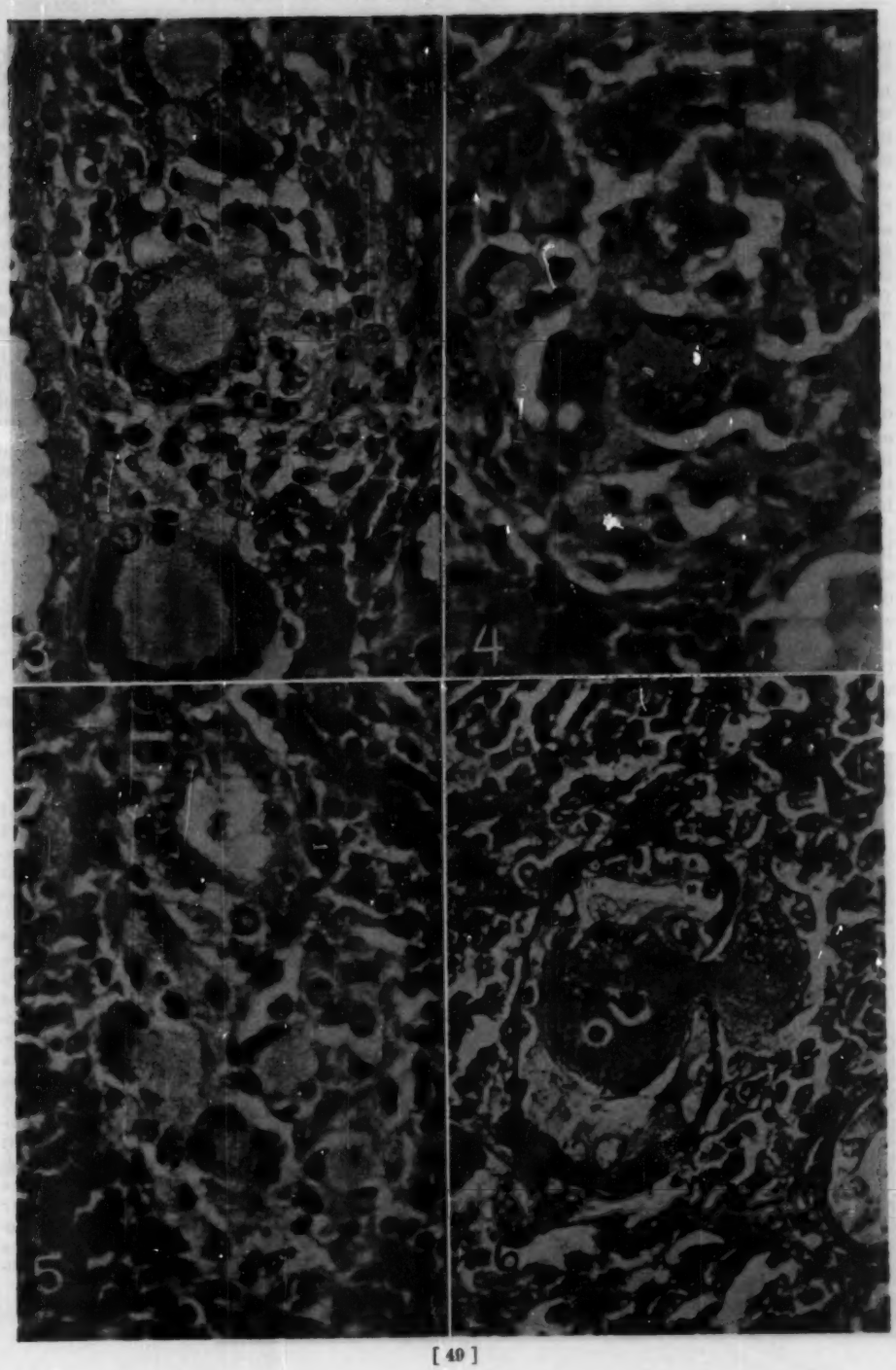
Fig. 3—Section of kidney from cow 376 showing multinucleated giant cell formations in an area of intertubular exudate. Hematoxylin and eosin. x 460.

Fig. 4—Section of kidney from cow 375 showing syncytial aggregate of the type frequently seen in the interstitial infiltrate. Although its exact histogenesis was not apparent, origin from tubular cells was considered probable (see fig. 9). Hematoxylin and eosin. x 543.

Fig. 5—Section of kidney from cow 383 with a syncytium (A) of uncertain histogenesis around a tubule (B). Hematoxylin and eosin. x 543.

Fig. 6—Section of kidney from cow 375 showing renal tubule (A) surrounded by large mononuclear cells (B). Notice the defect in the basement membrane through which the multinucleated mass protrudes. Allochrome stain (Wratten filter, 58). x 543.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olus was present in each nucleus. The tubular lumens frequently were filled with multinucleated masses where the individual cell outlines were indistinct (fig. 8). In other tubules, the nuclei of the proliferated cells lacked normal polarity and thus caused the lining epithelium to appear irregularly arranged. Rarely, mitoses were seen in the otherwise obviously proliferating tubular cells. Mononuclear cells of the inflammatory exudate frequently were found within the masses of tubular cells. There appeared to be both atypical tubule regeneration where the basement membrane was intact and exuberant tubular cell proliferation where partial disruption or dissolution of the basement membrane allowed the new-formed cells to invade the interstitial exudate (fig. 9). Other tubules in the patches of inflammatory exudate exhibited various stages of degeneration and dissolution associated with marked thickening of the basement membrane. Some showed frank coagulation necrosis. Casts of inflammatory cells and debris were present in some tubules both in, and adjacent to, the infiltrated areas. In most cases, the tubules outside of the areas of infiltration were normal or showed only mild regressive changes. Hyaline eosinophilic casts were present in some of these tubules. An occasional tubule was noticeably dilated and lined with a thin layer of epithelium.

The glomeruli generally were normal. Those surrounded by the cellular infiltrate frequently were atrophic and the capsular basement membrane was thickened and wrinkled. In only one kidney did a number of the glomeruli show increased cellularity, but there appeared to be no encroachment on the patency of the capillaries. Rarely the capsular space contained a small amount of pink-staining amorphous material.

Fibrosis was apparent in about a third of the cases. It was never extensive and was associated with the larger areas of interstitial infiltrate. With the several special stains, much of the fibrosis appeared to be due to a condensation of existing stroma rather than any appreciable proliferation of the fibrous connective tissues.

Small particles of hemosiderin were demonstrated in nearly all of the kidneys. The pigment was found most often in the epithelial cells of those tubules surrounded by the cellular exudate. Small amounts were present also in some of the macrophages of the interstitial exudate. The deposits in either instance were never extensive. The

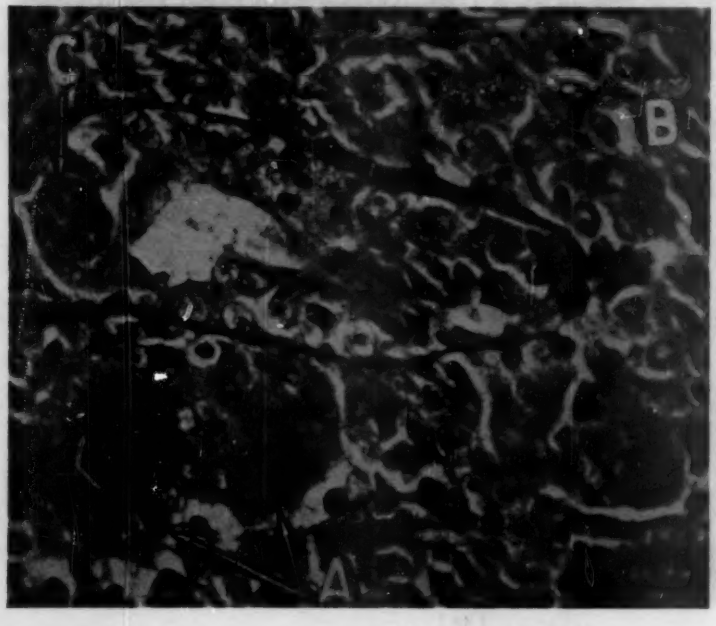

Fig. 7—Section of kidney of cow 375 showing renal tubule with an intact basement membrane and showing proliferative changes in the epithelial cells. Notice the two giant cells (A) and the syncytium (B) apparently arising independently of the tubular cells. The dark round body (C) in the tubular epithelium at the left had tinctorial properties similar to the large intratubular mass present in figure 6. Allochrome stain (Wratten filter, 58). x 543.

papilli of every kidney examined showed variable-sized, irregular-shaped, homogeneous, sometimes laminated, purple staining (hematoxylin and eosin) masses in the interstitial tissue and around the walls of capillaries. In the Prussian blue preparations, these masses stained grayish blue and frequently showed deeper staining peripheral portions. They stained deep purple-red with the periodic acid-Schiff method and did not appear to contain calcium. Rarely, small areas of calcification were found in the interstitial tissue of the cortex.

Unequivocal leptospiras were demonstrated in sections of kidneys from 6 cows. The organisms were free in the lumens of the cortical tubules and on the luminal border of the tubular cells, where some appeared to be partially within the tubular epithelium (fig. 10, 11, and 12). Typical complete leptospiras, as well as numerous fragmented forms, were seen. None was found in the interstitial tissue or in areas of the inflammatory exudate. It should be emphasized that the organisms were not numerous. One to four tubules in any one section were found to have from 3 to 20 complete leptospiras per tubule. Every oil-immersion field (x 970) of at least two large sections, which included cortex and medulla, from each kidney specimen was examined before the specimen was considered negative for leptospiras. Microorganisms other than the leptospiras were not seen in sections prepared by the acid-fast, buffered azure-eosinate, and periodic acid-Schiff stains.

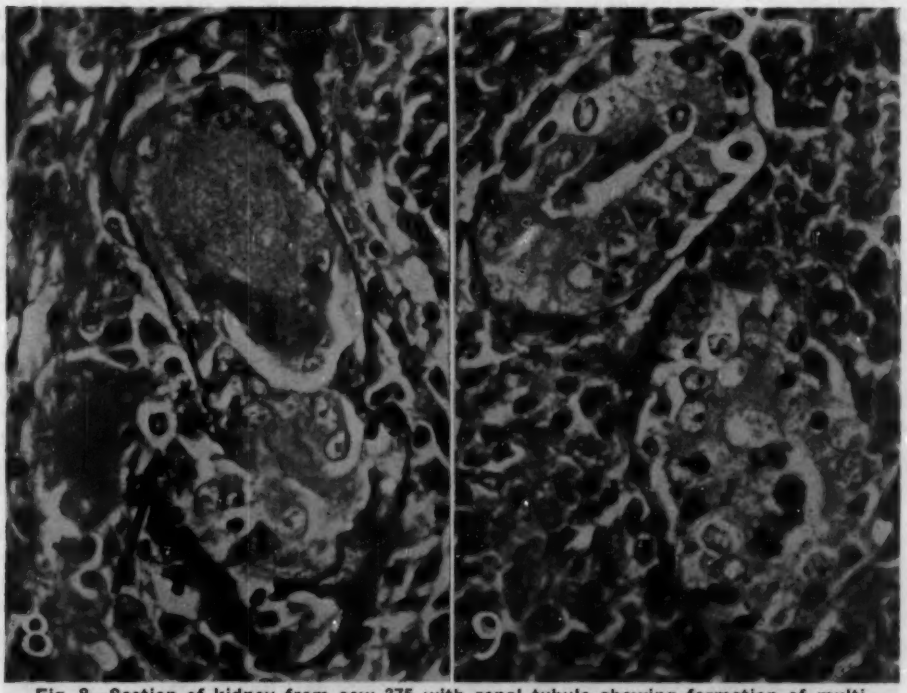

Fig. 8—Section of kidney from cow 375 with renal tubule showing formation of multinucleated cell masses from the tubular epithelium. The epithelial cells can be seen proliferating through a small defect in the basement membrane at the lower left side (arrow). Allochrome stain (Wratten filter, 58). x 543.

Fig. 9—Section of kidney from cow 375 with proliferative changes in two renal tubules entrapped in the cortical infiltrate. The basement membrane is thin but intact around the upper tubule. The basement membrane of the lower tubule has almost entirely disappeared. Allochrome stain (Wratten filter, 58). x 543.

Spleen.—The most prominent microscopic finding in the spleen was hemosiderosis, which was present in all specimens (fig. 13). The degree of pigmentation was regarded as slight in two, moderate in nine, and marked in four. The iron pigment appeared as dense masses and as fine particles scattered in the pulp. While much of the pigment was found in heavily laden macrophages, considerable amounts were present extracellularly in the meshes of the pulp reticulum. Although the hemosiderin was seen easily in the routine sections, its exact extent was appreciated best in the Prussian blue preparations. In all cases, the quantity of demonstrable iron pigment was considerably greater than that found normally in the spleens of some adult cattle.

The splenic lymph follicles were generally well developed. Reaction centers were present in many of the follicles but were prominent in only six specimens. Central necrobiosis was observed in some of the follicles and occasionally where there was loss of cells the centers presented a rarefied appearance. Hemorrhage was present in others. Perifollicular hyperemia associated with accumulations of eosinophilic and neutrophilic leukocytes was a constant finding. Frequently, the peripheral zone of the follicle was obscured by the infiltrating cells. Occasionally, the eosinophilic and neutrophilic leukocytes were abundant in small foci infiltrating the pulp. At times, focal hemorrhage was seen in the pulp. Rarely, a megakaryocyte was found. However, no definite foci of extramedullary hemopoiesis were identified. Except for hyperplastic changes in several specimens, the pulp was otherwise not altered. In one spleen, with prominent hyperplasia, there was a diffuse increase in large mononuclear cells and plasma cells in the pulp spaces. Fresh hemorrhage, especially periarterial, was present in the trabeculae in half of the cases. The vessels and capsule were not unusual. No leptospiras were found in silver impregnated sections.

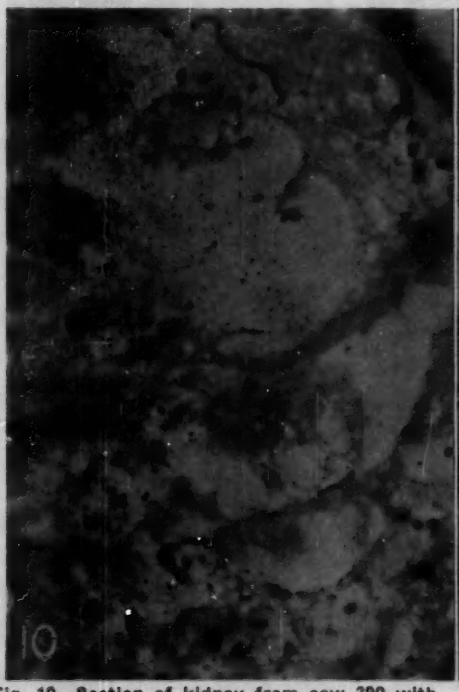

Fig. 10—Section of kidney from cow 390 with leptospiras in a subcapsular tubule. Parã technique. x 1,500.

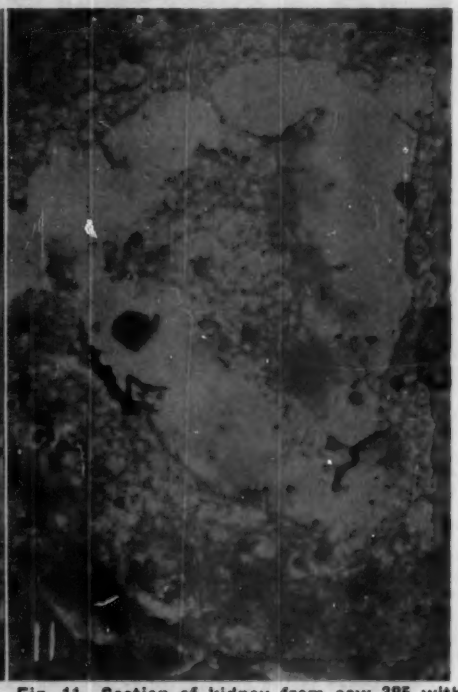

Fig. 11—Section of kidney from cow 385 with leptospiras in a cortical tubule. Parã technique. x 1,500.

Liver.—Microscopically, there were minimal changes in the liver. The hepatic cords and lobular arrangement were not disturbed. In all specimens, the cytoplasm of the hepatic cells appeared granular and somewhat hydropic. Mild fatty metamorphosis was present in one specimen. Most of the liver sections showed a variable but never marked portal and interlobular infiltration by lymphocytes, histiocytes, a few neutrophils, and an occasional plasma cell. Much of the portal infiltrate was concentrated around the biliary passages (fig. 14). Although the bile duct epithelium was generally intact, it was completely destroyed by the infiltrating cells in an occasional duct of one liver. An apparent increase in portal connective tissue was observed in 2 cases. Variable-sized foci composed of lymphocytes, histiocytes, and a few neutrophils were present in the lobules of the 11 specimens. The involved hepatic cells frequently were necrotic, especially in the larger cellular aggregates (fig. 15). Several sections showed an occasional small area of coagulation necrosis with a slight infiltration of neutrophils and lymphocytes. A small thrombus composed of fibrin and leukocytes was found in one interlobular vein adjacent to such an area of necrosis. Interlobular fibrosis was seen in one liver where it was associated with fibrous thickening of Glisson's capsule. Sections prepared by Perls' acid ferrocyanide method revealed no hemosiderin or only an occasional Kupffer cell with iron pigment, except in one liver. However, even in the latter section only minimal amounts of pigment were found in scattered areas. Leptospiras were not demonstrated in silver impregnated sections.

Uterus.—Two uteri showed the endometrial changes of pregnancy. No significant or specific changes were found in the remaining nongravid specimens. Iron-containing pigment in macrophages and extracellularly in the lamina propria, especially around the deeper uterine glands, was present to some extent in all specimens.

Lung.—Few microscopic changes were found in the sections of lung. Varying degrees of atelectasis and emphysema were observed in every case. In some areas the alveolar septums appeared somewhat thickened and, occasionally, a focus of lymphocytes and histiocytes was seen.

Discussion

The histopathological findings in the 15 cows reported in this paper conform in general with those reported by Mathews [7] for chronic leptospirosis in cattle. However, several points are worthy of comment in view of our observations.

Mathews [7] found extensive patchy lymphocytic interstitial infiltration in the kidneys from spontaneous cases of six weeks' to two months' duration. Similarly, Baker and Little,[2] and Ungar and Bernkopf [13] observed widespread focal interstitial nephritis in cows and/or calves for almost two months following experimental inoculation with leptospiras. Our observations would seem to indicate that the renal inflammatory lesions may be found for even longer periods since some of the cows in the reported group undoubtedly became infected

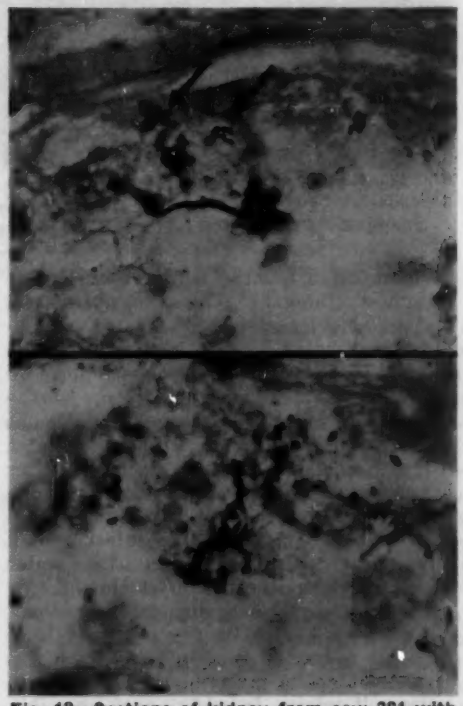

Fig. 12—Sections of kidney from cow 381 with complete and fragmented forms of leptospiras in different parts of a cortical tubule near the corticomedullary junction. Some of the leptospiras appear to be within the epithelium (arrows). Parã technique. x 1,500.

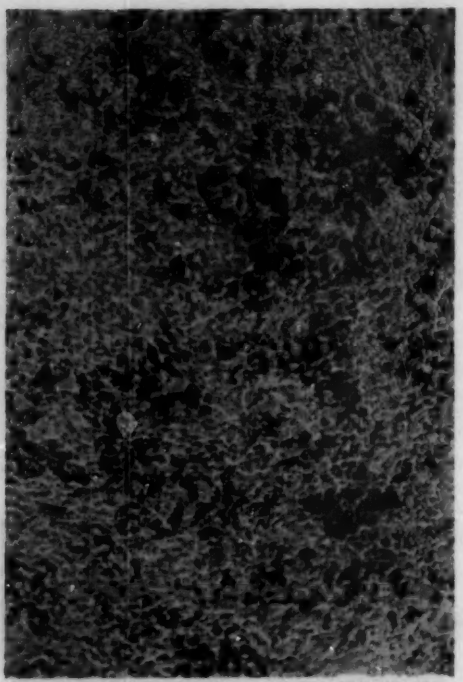

Fig. 13—Section of spleen of cow 382 showing deposits of hemosiderin in the pulp. Perls' Prussian blue (Wratten filter, 15). x 122.

at least three months before the time of slaughter (table 1). The syncytial masses and giant cells in many of the more extensive areas of interstitial exudate have not been mentioned in previous reports on either the acute or chronic forms of the disease. However, recently, similar lesions in the kidney were found in a cow and a calf six and seven weeks, respectively, after experimental inoculation with *L. pomona* while intertubular giant cells were found in a calf 174 days after inoculation with leptospiras.[11] The precise conditions under which the lesion occurs in cattle infected with leptospiras are not entirely clear. Nevertheless, much of the unusual tissue response appeared to result from the formation of epithelial syncytia of renal tubular cell origin. In addition, the possibility remains that some of the focal cellular masses represented a type of foreign body reaction around dead or damaged tubules. Such a distinction, however, could not be made in routine preparations. We are not aware to what extent these changes have been observed in the bovine kidney under other circumstances of tissue alteration.

Regeneration of the renal tubular epithelium in chronic bovine leptospirosis was reported by Mathews.[7] Details of the regenerative pattern were not presented and no mention was made of seeing the exuberant tubular cell proliferation such as we observed. Mathews expressed the belief that the regeneration of the renal tubules explained the recovery of many grave cases of the disease. By contrast, much of the epithelial proliferation in our specimens, at best, appeared to be only abortive attempts at tubule repair. Moreover, the areas of damaged parenchyma did not seem to be sufficiently extensive to cause appreciable altered renal function. At this point, it should be mentioned that the usual histological concept of tubular regeneration has been shown to be meaningless from the standpoint of restoration of the nephrons—structures essential to normal renal function.[8] Unfortunately, we have no clinical or laboratory data relative to the renal functional status of the cows studied.

Although small amounts of hemosiderin were seen in the kidneys, the spherical or oval, faintly eosinophilic, noniron-containing bodies in the tubular epithelium described by Mathews were not recognized. He found these peculiar pigmented bodies in both the acute and chronic forms and Ungar and Bernkopf [13] observed them in the acute disease.

Our finding leptospiras in as many as 6 cows was quite unexpected considering the inherent limitations of the method used and the chronicity of the disease. The observation is thought to have some epizootiological significance since several cows, in whose kidneys leptospiras were demonstrated, must have been infected for at least three months prior to the date of slaughter, as already mentioned. Previously, Baker, and Little,[2] and Ungar and Bernkopf [13] found that leptospiras persist in the kidneys for nearly two months. Also, York [15] stated that leptospiras may be shed in the urine for at least ninety days after the organisms localize in the kidneys near the end of the febrile period. Our observations re-emphasize the importance of the chronic renal carrier in the epizootiology (and epidemiology) of the disease.

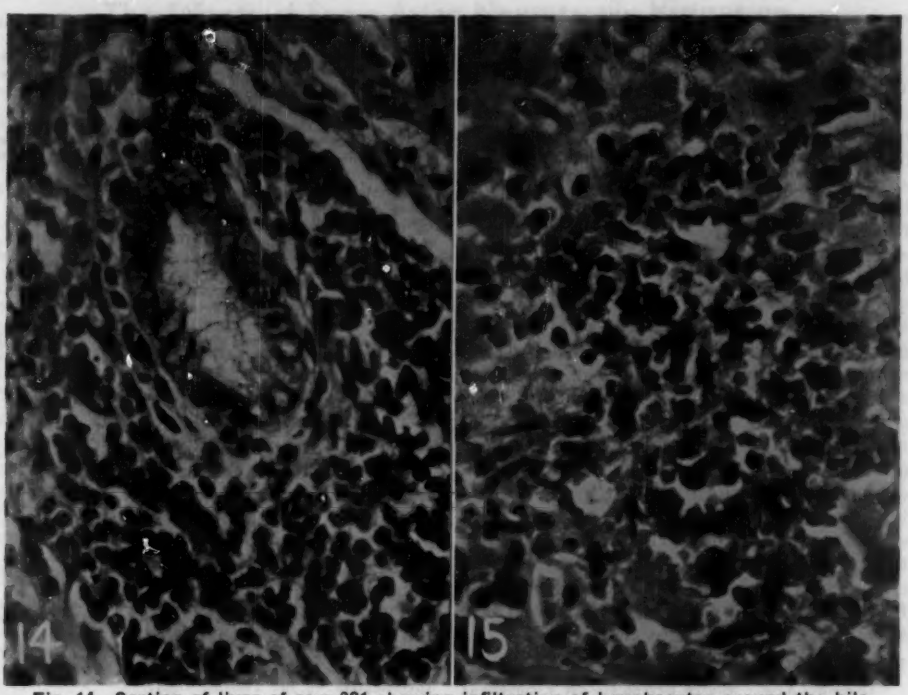

Fig. 14—Section of liver of cow 381 showing infiltration of lymphocytes around the bile duct in a portal space. Hematoxylin and eosin. x 500.

Fig. 15—Section of liver of cow 376 showing small focus of hepatic cell loss accompanied by hemorrhage and an aggregation of lymphocytes with an occasional neutrophil. The surrounding liver cells are not appreciably altered. Hematoxylin and eosin. x 500.

Undoubtedly, the accumulation of iron pigment in the spleen is subject to considerable variation depending upon the severity of the hemolytic changes accompanying the acute signs of the infection. The rather extensive hemosiderosis observed in most of the spleens was considered a reflection of marked antecedent erythrocyte destruction. Mathews found marked splenic hemosiderosis as well as atrophy of the splenic lymphoid tissue. The latter change was not observed in our material.

Periportal and interlobular mononuclear cell infiltration is often found in the liver during the acute phase of leptospiral infections.[1,3,11,13] Mathews did not mention the lesion in his description of chronic leptospirosis. The tendency for the exudative lesion to be a pericholangitis was quite evident in our specimens even though collected late in the course of the disease.

The lack of significant microscopic changes in the uteri is of interest in view of the impression of some observers that about a third of the cows which retain the fetal membranes following abortion due to leptospiral infection prove to be barren. It must be admitted, however, that a more detailed pathological examination of the genitalia would be necessary to ascertain whether there is any morphologically demonstrable basis for the impaired fertility in these cows.

As yet it is not apparent to what extent the infecting strain or serotype of Leptospira influences the pathological pattern of the disease. Additional clinicopathological and laboratory observations should contribute to the elucidation of this point and then permit comparisons which at present seem premature in view of the incompleteness of the existing data.

SUMMARY

The histopathological findings in several organs of 15 Hereford cows from a herd spontaneously infected with *Leptospira pomona* are described. The most prominent finding in the kidneys was a widespread chronic focal interstitial nephritis. Renal tubular cell proliferation was a common finding. Where the latter change was associated with focal defects in the tubular basement membrane or with its almost complete dissolution, the proliferating tubular cells formed bizarre syncytia and giant cells in the larger areas of interstitial exudate. Hemosiderosis was the most constant finding in the spleen. Variable, but never marked, portal and interlobular mononuclear cell infiltration was the most conspicuous lesion in the liver. No significant changes were found in the uterus and lungs. Unequivocal leptospiras were demonstrated in sections of kidney from only 6 of the 15 cows. These findings are discussed in view of the reported microscopic findings in cattle studied during the late stages of leptospiral infection.

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The Effects of Some Avian Neurotropic Viruses on Young Dairy Calves

C. F. HELMBOLDT, D.V.M., Ph.D.; R. E. LUGINBUHL, M.S.; A. H. HAMMAR, M.S.; S. F. SATRIANO, M.S.; E. L. JUNGHERR, Vet. Dipl., D.M.V.

Storrs, Connecticut

IT IS a common farming practice to allow the association of dairy animals and fowl such as poultry and pheasants. The possibility, therefore, exists that diseases of one class may become established in members of the other class. This was illustrated by the spontaneous case of Newcastle disease in a young dairy animal reported by Yates *et al.*[28] The present communication deals with results obtained when the viruses of avian encephalomyelitis (AE), eastern equine encephalomyelitis (EEE), and Newcastle disease (ND) were inoculated into dairy calves.

REVIEW OF LITERATURE

Avian Encephalomyelitis.—Attempts to adopt this virus to common laboratory animals have met with failure.[24]

Eastern Equine Encephalomyelitis.—This disease occurs commonly in horses and mules.[23] Experimentally, it has been produced in calves, dogs, mice, monkeys,[12] rabbits, and guinea pigs with the last being the most readily affected.[6] These animals sickened in a few days with symptoms referable to a central nervous system involvement. Histological examination of the horse brain revealed foci of neutrophils and perivascular cuffs of lymphocytes and neutrophils.[6, 12] The primary lesion was considered to be neuronal necrosis associated with neuronophagia and vacuolation of the Nissl substance.[6] However, this was not a universal finding as some investigators reported little neuronal damage.[10] Further study revealed that neutrophils tended to be replaced by mononuclear cells after the second day of illness.[20] The lesions were primarily located in the gray matter in all animals but varied among the species; for example, the rabbit sustained maximum injury in the hippocampus and rhinencephalon.[6] Calves differed only in that the changes were more intense in the cerebral cortex and were characterized by more acute necrosis than in the horse and neutrophils were less common particularly in the perivascular cuffs.[6] No difficulty was reported in recovering the virus from the experimental hosts.

Newcastle Disease.—This disease has been produced experimentally in various animals which include the calf,[2] cotton rat,[22] guinea pig,[14] Syrian hamster,[14–17, 20, 21] hog,[5] rhesus monkey,[18, 25] sheep,[26] short-tailed shrew,[19] and Swiss albino mice.[5, 7, 9, 11, 14] Spontaneous mammalian cases are apparently rare; aside from human cases, only 1 case has been reported in a dairy calf.[28]

The California ND virus strain 11,914, passaged 320 times intracerebrally in the Syrian hamster, has become less pathogenic for chicken embryos but has increased in virulence for hamsters[20] with the result that the intranasal,[16] intratesticular,[2] intradermal,[2, 17] rectal,[21] and oral routes could cause the characteristic syndrome of irritability, malaise, and paralysis within a few days.

The monkey (*Macaca mulatta*) proved susceptible to intracerebral inoculation of the Manhattan and California 11,914 strains. The animals became acutely ill in five to ten days with symptoms of ataxia, weakness, fever, and gross tremors but recovered rapidly as a rule. Brain material up to the third passage from ill monkeys caused symptoms when injected into the other monkeys. Histological studies revealed encephalitis limited largely to the brain stem, chorioiditis, meningitis, and myelitis confined to the gray matter of the cervical region. A sterile abscess invariably formed at the site of the injection.[25]

The California strain 11,914 and the Australian strain were successfully passaged in Swiss albino mice. They cause symptoms of circling, convulsions, and hyperirritability in two to eight days.[5] The strains tended to lose pathogenicity for chicks after several passages.[9] Histological examination of the murine brain revealed generalized capillary congestion and focal loosening of the ground substance with mobilization of microglia. Coagulation necrosis and shrinkage of nerve cells were noted in the hippocampal gyrus, thalamus, and temporal lobe. Only minimal perivascular cuffs were seen. No lesions were observed other than those in the brain.[9]

Hogs generally died within a week when inoculated intracerebrally with the Iowa 125 strain. Although the virus was recovered from five of eight hog brains, hog-to-hog passage was unsuccessful. Sheep also died as a result of inoculation via the intracerebral route. However, some died in five days whereas others became ill only after a month. Sheep-to-sheep passage also failed although

From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

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Dr. Hammar is now with Lederle Laboratory, Pearl River, N. Y.

the virus was frequently recovered from the brain. There were no histological changes characteristic of the type seen in chickens.[5]

In the spontaneous case reported in the calf, the lungs were edematous and infiltrated with serosanguinous material particularly in the bronchi from which ND virus was isolated.[20] An experimental calf, inoculated intracerebrally, became ill within ten days with symptoms suggesting involvement of the central nervous system. No virus isolation nor histological examination was reported.[2]

MATERIALS AND METHODS

Twenty-five 2- to 3-month-old dairy calves of Holstein-Friesian, Guernsey, and Jersey breeds were used of which 6 served as controls. The following viruses were injected intrathecally or intracerebrally at dilutions of 10^{-1}, the diluent being either buffered saline or tryptose broth (table 1); AE, strain A22,557, which had a minimum chicken infective dose of 10^{-6} per 0.03 ml. (333,333 units) infected brain at the fourteenth chicken passage; EEE, strain A24,939, which had an embryo minimum lethal dose (e.m.l.d.$_{50}$) of 10^{-6} per 0.2 ml. (500,000 units) allantoamniotic fluid (AAF) at the fifteenth embryo passage; ND, strain GB, which had an e.m.l.d.$_{50}$ of 10^{-6} per 0.2 ml. (50,000,000 units) AAF at the fifteenth embryo passage. In addition, 2 calves were inoculated with a brain suspension 10^{-1} in buffered saline from a calf dying of ND and 1 calf was injected with normal calf brain suspension 10^{-1} in buffered saline. A total of 1 ml. was injected in each instance.

Intrathecal injections were made at the suboccipital space[8] with the calf in a standing position; no anesthesia was necessary as it was found that the 3-in. 18-gauge needle employed caused little discomfort. Intracerebral injections were done under general anesthesia induced by intravenous use of chloral hydrate, 4.62 gr. and magnesium sulfate, 3.86 gr. per milliliter of distilled water; between 30 and 50 ml. were required. A "handy drill" with a No. 6 dental burr was used to trephine the right frontal bone about 1 inch laterally to the midline and 1 inch anterior to the poll. Animals were held for one month or more following inoculation, unless death or impending death intervened, then were destroyed for necropsy. Prior to inoculation and at weekly intervals postinoculation, blood specimens were obtained for evidence of viremia and for serum neutralization tests. At necropsy a portion of the left noninoculated cerebral hemisphere was removed and stored at —10 C. until it was suspended in tryptose 10^{-1} and injected into 10 day-old chicken embryos or chicks. Spinal fluid was withdrawn and handled in the same manner save for a portion which was used for cell counts. For histological study, the brain and portions of the

TABLE 1—Results of Inoculations Into the Central Nervous System (CNS) of Young Dairy Calves

Breed	Inoculation: mode	Inoculation: virus	Inoculation: diluent	Incubation in days	CNS symptoms	Dead or dying	Leukocytes in spinal fluid per cmm.	Final blood titer	Chicken embryo deaths	Neuropathological changes
VIRAL INOCULATES										
Guernsey	IT	AE	Saline	—	—	—	<5		Not done	—
Guernsey	IC	AE	Saline	—	—	—	10		Not done	—
Jersey	IC	EEE	Saline	1	+	+	50	10^{-1}	6/6	+
Jersey	IC	EEE	Saline	1	+	+	40	10^{-2}	6/6	+
Guernsey	IT	ND	Tryptose	1	+	+	100	10^{-2}	0/6	+
Guernsey	IT	ND	Tryptose	1	—	—	<5	10^{-1}	0/6	—
Guernsey	IT	ND	Tryptose	1	—	—	<5	10^{-2}	0/6	—
Guernsey	IT	ND	Saline	—	—	—	<5	10^{-1}	0/6	—
Guernsey	IT	ND	Saline	—	—	—	<5	10^{-2}	0/6	—
Guernsey	IC	ND	Saline	14	+	+	Not done	10^{-2}	0/6	+
Guernsey	IC	ND	Saline	14	+	+	100	10^{-2}	0/6	+
Holstein-Friesian	IC	ND	Saline	14	+	+	16		0/6	+
Holstein-Friesian	IC	ND	Saline	14	+	+	<5		0/6	+
Guernsey	IC	ND	Saline	1	+	+	Not done	10^{-2}	0/6	+
Holstein-Friesian	IC	ND	Saline	—	—	—	30	10^{-2}	0/6	+
Holstein-Friesian	IC	ND	Saline	—	—	—	22	10^{-2}	0/6	+
Holstein-Friesian	IC	ND	Saline	—	—	—	<5		0/6	+
Holstein-Friesian	IC	ND calf brain	Saline	—	—	—	<5	10^{-1}	0/6	—
Holstein-Friesian	IC	ND calf brain	Saline	—	—	—	<5	10^{-1}	0/6	—
CONTROLS										
Guernsey	IT	—	Tryptose	—	—	—	<5	10^{-1}	0/6	—
Guernsey	IT	—	Saline	—	—	—	<5	10^{-2}	0/6	—
Holstein-Friesian	IC	—	Saline	—	—	—	Not done		0/6	—
Holstein-Friesian	IC	—	Saline	—	—	—	Not done	10^{-2}	0/6	—
Jersey	IC	Allantoic fluid	Saline	—	—	—	Not done	10^{-2}	0/6	—
Holstein-Friesian	IC	Normal calf brain	Saline	—	—	—	<5	10^{-1}	0/6	—

AE—avian encephalomyelitis; EEE—eastern equine encephalomyelitis; IC—intracerebral; IT—intrathecal; ND—Newcastle disease; ND calf brain—brain material from calf dying of ND; 0/6—numerator denotes number of deaths; denominator the number of eggs inoculated.

cervical, thoracic, and lumbar cord were fixed in 10 per cent formalin. The viscera were similarly handled. Tissue blocks of the brain were taken from the corpus striatum, thalamus, hippocampus, corpora quadrigemina, pons, medulla oblongata, cerebellum, cerebrum at site of injection, and cerebrum opposite the site of the injection. Tissues were imbedded in paraffin and stained with hematoxylin-triosin.

RESULTS

Avian Encephalomyelitis.—The 2 calves inoculated (table 1), either by the intrathecal or the intracerebral route, did not become ill or react in any way. Histological examination of the brain and spinal cord failed to reveal significant changes. The leukocyte count of the spinal fluid was less than a dozen cells per cubic millimeter. Attempts to recover the virus by intracerebral inoculation of calf brain material suspended in buffered saline 10^{-1} into 28 day-old chicks failed to produce illness. It was concluded that AE virus is incapable of adapting itself to existence in the central nervous system of the dairy calf.

Eastern Equine Encephalomyelitis.—Three Jersey calves were employed for this phase of the experiment. One calf, serving as a control, received sterile allantoic fluid from a normal chicken embryo. Two calves were inoculated (table 1) with the allantoic fluid of chicken embryos dying of EEE. On the first postinoculation day, the calves developed a 2 to 3 degree rise in body temperature, and on the second postinoculation day, stiffness, lethargy, anorexia, and continued fever were observed. The disease progressed rapidly so that on the third day the calves were semicomatose and were killed at the height of the febrile reaction. The control calf acted normally for thirty days and showed no lesions on necropsy.

Histological examination of the central nervous system of the inoculated calves revealed a moderate diffuse encephalomyelomeningitis. The reactive cells were chiefly lymphocytes and mononuclears with neutrophils uncommon or absent. Vascular changes in addition to the perivascular lymphocytic infiltrate were expressed by endothelial damage and frequent accumulations of erythrocytes. Perivascular edema was likewise seen (fig. 1). Unlike reports for the horse,[6,10] the lesions were present in equal intensity in the white matter and gray matter. All examined portions of the brain and cord showed involvement.

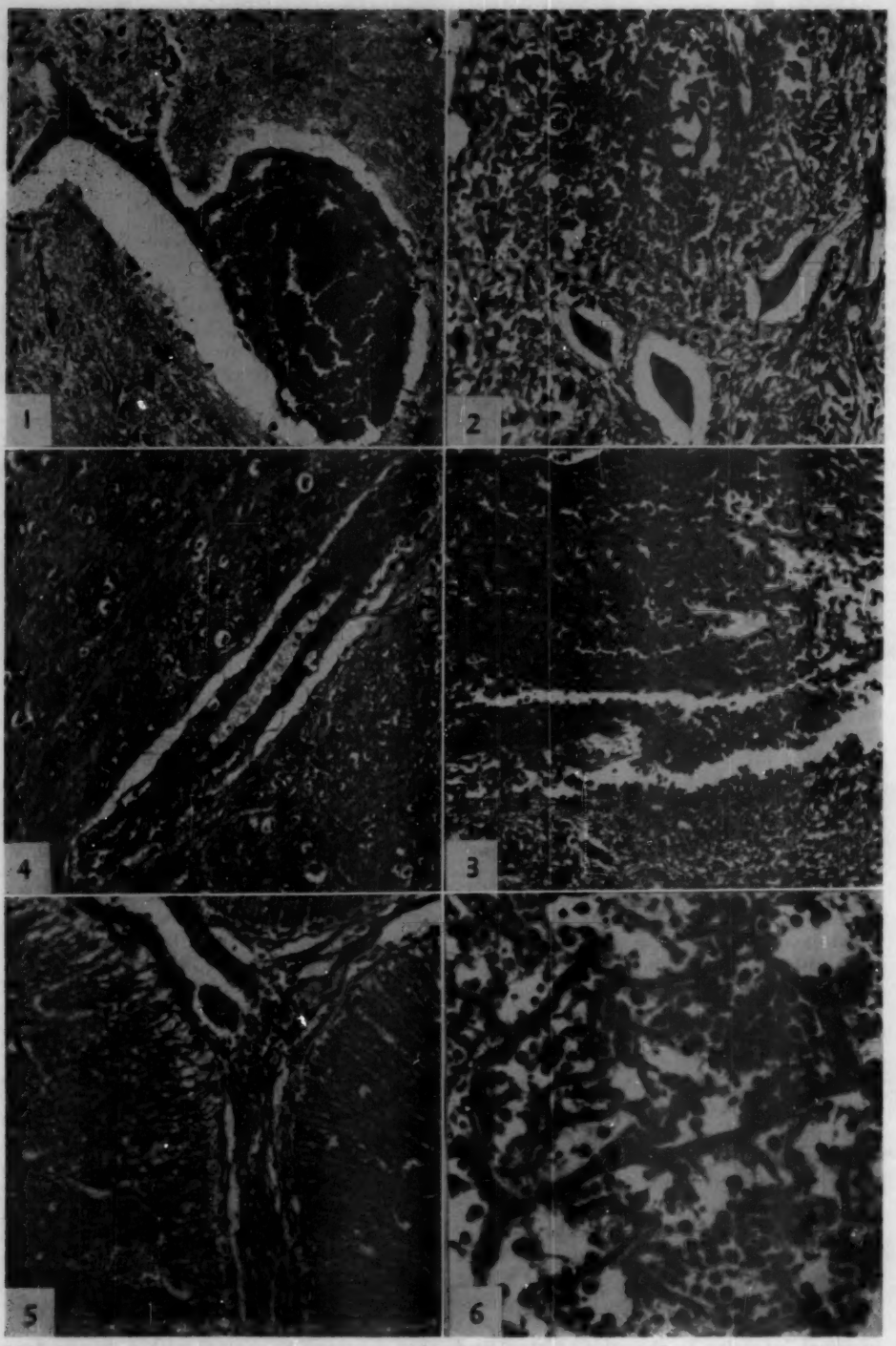
One of the authors (ELJ) observed the result of the intrathecal injection of this strain of the virus into a 15-year-old gelded Thoroughbred which succumbed after two days of a typical clinical picture of the disease. Neuropathological lesions resembled those of the calf except that more neutrophils were seen in the cuffs and reactive foci in the equine brain. In neither the equine nor the bovine brain was neuronal damage a prominent feature. Frequently, it was noted in the calf that neurons were apparently normal even though a paraneuronal edema existed. This was seen particularly in reactive foci (fig. 2).

The virus was easily recovered from the brain of dying calves, 10-day-old chicken embryos being consistently killed within one to five days when inoculated via the chorioallantoic sac with 0.2 ml. of a 10^{-1} inoculum of calf brain suspended in buffered saline.

Newcastle Disease.—A total of 20 animals was used in this phase of the work. Six animals received the virus via the intrathecal route (table 1) with varying results.

When tryptose broth was used as the diluent in three instances, undesirable effects followed. The first postinoculation day was marked by fever, anorexia, lethargy, and reluctance to move but by the third day 2 had completely recovered while the third had become prostrate and was killed *in extremis.* Histological examination of the latter animal revealed marked purulent meningitis of the cervical cord only at the site of inoculation (fig. 3). Since bacteriological and virological studies of the brain, spinal cord, and spinal fluid from this calf failed to indicate infection, it was presumed that the meningeal reaction was in the nature of an allergic phenomenon. This may also have been responsible for the fleeting febrile reaction in the other 2 calves since tissues and spinal fluid failed to reveal abnormalities on necropsy one month after inoculation.

In consequence of these results, 2 calves were given ND virus in buffered saline and 1 received tryptose broth only, all by the intrathecal route (table 1). One of the calves receiving the virus exhibited a 1 degree rise in temperature the following day but returned to normal within twenty-four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hours and remained so from there on. Thus when ND virus suspended in tryptose broth was injected, a definite reaction occurred whereas the virus suspended in saline or tryptose broth alone produced no reaction. With the exception of the leptomeningitis mentioned above, none of the animals injected by the intrathecal route revealed histopathological lesions, or bacteriological or virological agents.

Of 8 calves receiving ND virus suspended in saline, via the intracerebral route (table 1), all but 1 recovered without febrile or neurological reactions. That 1 developed a temperature of 105 F. which subsided within a few days, but then the calf became increasingly weak, refused food, lapsed into a coma, and was killed on the tenth day postinoculation. Necropsy revealed a subdural hematoma extending over the entire right cerebrum, the side inoculated. This hemorrhagic lesion was probably of traumatic origin initiated by faulty operative technique. An abscess, later found to be sterile, of 0.5 cm. diameter was present at the site of the injection. The remaining 7 calves continued in excellent health until the fourteenth day postinoculation at which time changes came with dramatic suddenness. One calf died during the night although it was clinically normal the previous evening, another was found in a semicomatose state with frequent convulsions, while 2 others showed slight incoordination and hyperesthesia. The mere clapping of hands induced in the latter frantic dashes about the pen lasting for several minutes, invariably followed by a period in which the animal stood in a braced position, trembling violently. Within two days after the onset of the symptoms, the animals were paralyzed and moribund and were killed. Thus of the original 8 animals subjected to intracerebral inoculation of the ND virus only 3 survived; the latter remaining in apparently good health until the thirtieth day postinoculation at which time they were killed.

Histological examination of the central nervous system of these 8 calves revealed unexpected findings. The brains of the apparently healthy calves, as well as those succumbing to the virus, presented slight to moderate encephalitis, nonpurulent and diffuse in character usually accompanied

Legends for Illustrations on Opposite Page

All figures are photomicrographs of bovine brain sections embedded in paraffin and stained with hematoxylin-triosin.

Fig. 1—Cross section from corpora quadrigemina of a 2-month-old Jersey calf injected intracerebrally with eastern equine encephalomyelitis virus suspended in saline. The calf was killed in extremis. A large perivascular cuff with some disintegrating endothelial cells lies in an edematous area. Numerous erythrocytes and lymphocytes make up the cuff. x 185.

Fig. 2—Cross section of spinal cord, midthoracic region, from a 2-month-old Jersey calf injected intracerebrally with eastern equine encephalomyelitis virus suspended in saline. This calf also was killed in extremis. The photomicrograph embraces the tip of a ventral horn. Several apparently normal neurons appear in intimate contact with a glial focus. Arrow points to a degenerated neuron. x 185.

Fig. 3—Cross section of spinal cord, cervical region near site of injection, from a 3-month-old Guernsey calf injected intrathecally with Newcastle disease virus suspended in tryptose broth. Calf was killed in extremis. The leptomeninges are infiltrated by neutrophils. A small portion of the dorsal spinal cord is visible. x 145.

Fig. 4—Cross section from corpora quadrigemina of a 3-month-old Holstein-Friesian calf injected intracerebrally with Newcastle disease virus suspended in saline. A perivascular cuff of lymphocytes, typical of the reaction, is seen. This calf was killed on the sixteenth day postinoculation. x 145.

Fig. 5—Section of the cerebellum of a 3-month-old Guernsey calf injected intracerebrally with Newcastle disease virus suspended in saline. The calf, clinically normal, was killed on the thirtieth day postinoculation. The interfoliar pia mater is slightly infiltrated by lymphocytes. x 145.

Fig. 6—Cerebral cortical section at site of injection from a 3-month-old Guernsey calf injected intracerebrally with Newcastle disease virus suspended in saline. The calf died on the fifteenth day postinoculation. Compound granular corpuscles and proliferated capillaries occupy the necrotized area resulting from the inoculation. x 145.

by a slight leptomeningitis. Lymphocytes formed small perivascular cuffs (fig. 4) and, together with mononuclear cells, infiltrated the leptomeninges (fig. 5). Small glial foci often accompanied the vascular lesion. Although the alterations were present throughout the brain, their greatest concentration was in the mesencephalon. Generally, the spinal cord was spared but in two instances slight gliosis and perivascular cuffs were observed as far back as the midthoracic cord.

At the site of the injection, which could be seen grossly as a small ulcer, there was a large necrotic area undergoing repair. The lesion was essentially a mass of compound granular corpuscles in a loose network of proliferating capillaries (fig. 6). Such foci were considered to resemble the sterile abscesses reported by Wenner *et al.*[25] in their work with monkeys.

Attempts to recover the virus from the affected calves by inoculation of 10-day-old chicken embryos failed. It appeared that the ND virus in the calf brain acted as in the mouse brain; according to Kilham *et al.*,[9] the ND virus could establish itself in the murine brain but could not complete its life cycle to the point of reproduction.

Serum neutralization tests with heat inactivated serums from the exposed calves failed to show any significant rise in titers (table 1). Controls were found to have the same titer as those inoculated.

Two calves were given intracerebral inoculations of brain material from 1 of the calves which had succumbed to ND on the fifteenth day postinoculation. The brain was suspended in buffered saline 10^{-1} and treated with streptomycin-penicillin and injected into the calves. A third calf served as a control by being subjected to a similar procedure, except that the brain material used came from a normal calf (table 1). No abnormalities were observed clinically, and histological, virological, and serological studies were negative in all 3 calves.

Two calves served as controls by being subjected to intracerebral inoculations of buffered saline. They remained clinically normal and laboratory methods failed to detect any abnormalities.

Differential Diagnosis.—Experimental EEE was easily differentiated from ND in calves when symptoms, viral isolation, and histological examinations were considered.

Clinically, the calves inoculated with EEE virus presented a typical akinetic picture, being comatose, while the calves inoculated with the ND virus presented marked hyperkinesia, rarely remaining quiet in the face of external stimuli and having frequent convulsions when no longer able to stand. However, these symptoms might vary under different conditions. A reversal of symptoms was recorded in man in cases of epidemic encephalitis which occurred in Winnipeg, Canada.[1] In one outbreak the affliction was akinetic, but a few years later the same disease caused hyperkinetic symptoms.

Also the EEE virus could be isolated from the brain whereas the ND virus could not be recovered.

Histological differentiation was not sharply defined. Spinal cord lesions were common in the EEE cases, but were present in only 2 of the 8 ND cases while brain lesions of EEE were considered to be more widespread and severe than those of ND. Aside from being larger, the perivascular cuffs in EEE cases were often erythrocytic in nature. This may have been due to the necrotizing properties of this particular strain of the virus, a feature also observed in spontaneous cases of pheasants.[4,7] Generally speaking, the lesions of EEE tended to be more pronounced and diffuse than those of ND. In the calf brain, neutrophils were not common in the lesions and thus did not serve as an outstanding differential feature.

SUMMARY AND CONCLUSIONS

Twenty-five 2- to 3-month-old dairy calves were inoculated with viruses of avian encephalomyelitis, eastern equine encephalomyelitis, or Newcastle disease. Two calves, 1 injected intrathecally and 1 intracerebrally, with avian encephalomyelitis virus in saline continued in good health, the virus not being recovered nor histological changes observed.

Two calves were inoculated intracerebrally with the eastern equine encephalomyelitis virus in allantoic fluid in saline while a control received sterile allantoic fluid in saline. The 2 calves were killed *in extremis* on the third postinoculation day and virus was recovered from the brains which were found to present diffuse encephalomyelomeningitis. The control was normal.

Of 3 calves inoculated intrathecally with Newcastle disease virus in tryptose broth, 1 exhibited a severe reaction within forty-eight hours apparently due to an allergic leptomeningitis while 2 showed slight discomfort from which they recovered. The 2 calves given Newcastle disease virus in saline and the 1 given saline and tryptose broth showed no effect.

Of 8 calves injected intracerebrally with Newcastle disease virus in saline all remained asymptomatic until the fourteenth day at which time 1 died suddenly and 4 showed hyperesthesia and progressive paralysis. No specific serological reactions were observed, nor was the virus recovered from any of these calves. All calves including the 3 apparently healthy ones showed moderate nonpurulent diffuse encephalitis whereas the two controls, given saline intracerebrally, were normal.

No changes were seen in 2 calves injected intracerebrally with a brain suspension from 1 of the calves which had succumbed to the intracerebral injection of the Newcastle disease virus and 1 calf injected with a brain suspension from a normal calf.

In this study, the eastern equine encephalomyelitis virus caused akinetic symptoms whereas the Newcastle disease virus caused hyperkinesia.

It was concluded that: (1) avian encephalomyelitis virus is of no importance to the bovine species as it can not adapt itself to existence or reproduce in their system; (2) eastern equine encephalomyelitis can be of some danger to the bovine population as it not only can exist in this species but can reproduce itself; (3) Newcastle disease is of little importance to cattle since it can adapt itself for existence only when introduced under unusual circumstances and can not reproduce.

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Plasma and Milk Tocopherol Levels of Cows Compared with the Plasma Tocopherol Levels of Their Foster Calves

J. W. SAFFORD, D.V.M., and KARL F. SWINGLE, Ph.D.

Bozeman, Montana

PRACTICING veterinarians have observed both the skeletal and cardiac forms of muscular dystrophy in calves that were transferred (grafted) to cows after the cows' own calves had died with muscular dystrophy. If this could be repeated, it would be a way of obtaining data and observing the development of spontaneous muscular dystrophy in calves.

EXPERIMENTAL PROCEDURES

Three cows from three "muscular dystrophy" ranches in western Montana were obtained for the experiment.

Cow 5,068 was a 6-year-old Red Poll. Her calf was born Feb. 14, 1953. Another newborn calf was fed by this cow starting Feb. 15, 1953. Both calves had developed the skeletal form of muscular dystrophy by March 14.

Cow 5,093 was a 3-year-old Aberdeen Angus. Her calf died with the cardiac type of muscular dystrophy Mar. 26, 1953, at the age of 22 days.

Cow 5,150 was a 5-year-old Hereford. Her calf died with the skeletal form of muscular dystrophy April 19, at the age of 2 months.

Three male Jersey calves were obtained from two herds free of muscular dystrophy. The calves were permitted to obtain colostrum from their dams one day following birth. Calf 5,091 was placed on cow 5,068 on March 28; calf 5,094 on cow 5,093; and calf 5,151 on cow 5,150 on April 24, 1953.

The hays, that had been the entire ration of the cows the winter and spring preceding the loss of their calves with muscular dystrophy and for the specified time following the "graft" of the new calves, were described as follows:

Hay No. 1, fed to cow 5,068 the first forty-eight days of this experiment:

Quality: U. S. No. 2 alfalfa
Color: 35%
Leafiness: 35 to 40%
Foreign matter: Trace

Hay No. 2, fed to cow 5,093 the first forty-nine days of this experiment:

Quality: U. S. No. 2 grass hay
Timothy: 92%
Clover: 3%
Red top: 5%
Douglas sedge: Trace
Baltic rush: Trace
Canada Bluegrass: Trace
Kentucky Bluegrass: Trace

Hay No. 3, fed to cow 5,150 the first forty-three days of this experiment:

Quality: U. S. No. 3 alfalfa hay
Color: 35%
Leafiness: 10%
Foreign matter: 10%

After these hays were fed as indicated, a U. S. No. 1 alfalfa hay was fed cow 5,068 for the next thirty-two days; cow 5,093 for the next thirty-three days; and cow 5,150 for the next 11 days. On June 18, the cows and calves were turned to green pasture for nineteen days. Then the experiment was terminated.

Milk samples were taken daily from each cow for tocopherol determinations, which were made by methods described by Quaife.[1]

Blood samples were taken from each cow and calf at weekly intervals for tocopherol determinations. Blood plasma tocopherol determinations were made using methods described by Quaife and Harris.[2]

Clinical observations were made daily on each calf.

Electrocardiogram tracings were obtained from each calf at weekly intervals. A Sanborn "cardiette," No. 202, was used with tracings recorded from each of three leads.

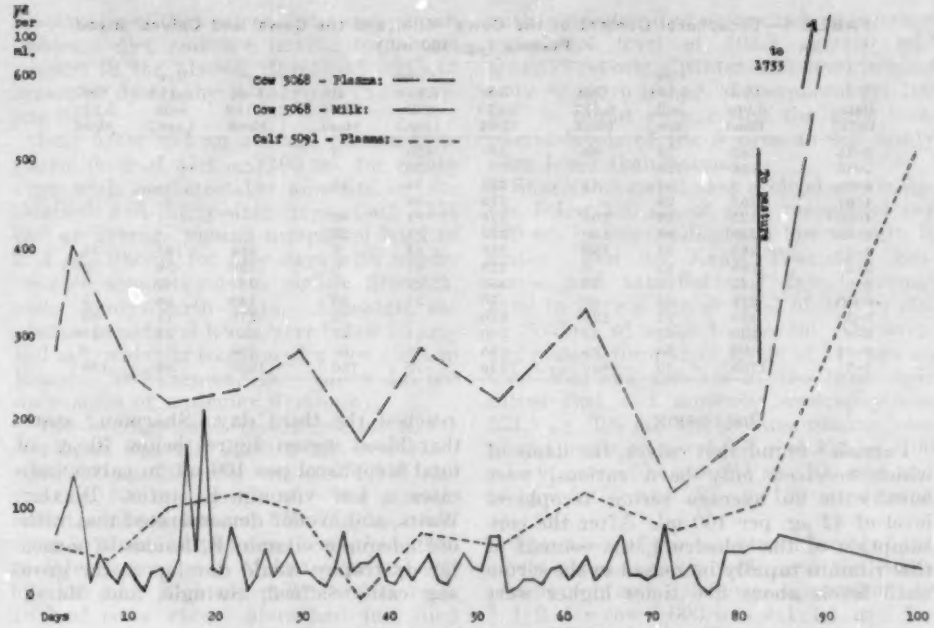
RESULTS

Clinical signs of muscular dystrophy were not observed in the 3 calves of this experiment.

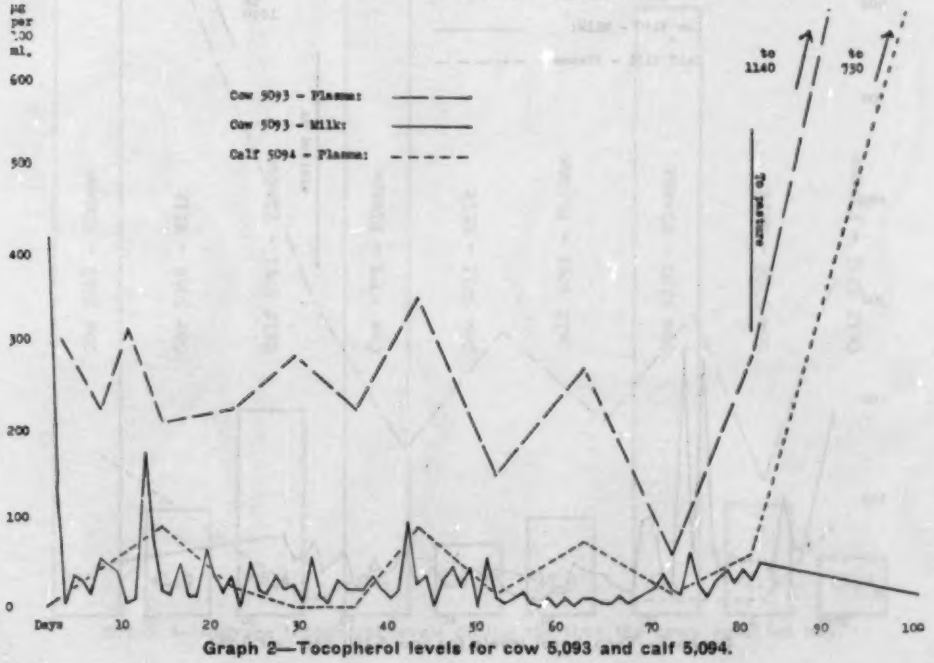
The duration of the intervals, the cycles, rates, Bazett's K, and the potentials of all waves on all leads in the electrocardiogram tracings made on the 3 calves were within normal limits as described by Lepeschkin.[3]

The results of the plasma and milk tocopherol analyses of the cows and calves are given in table 1. The relationships of the cows' plasma and milk tocopherol and the calves' plasma tocopherol levels are expressed in graphs 1, 2, and 3.

From the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating). Paper 344 journal series, Agricultural Experiment Station, Montana State College, Bozeman.



Graph 1—Tocopherol levels for cow 5,068 and calf 5,091.



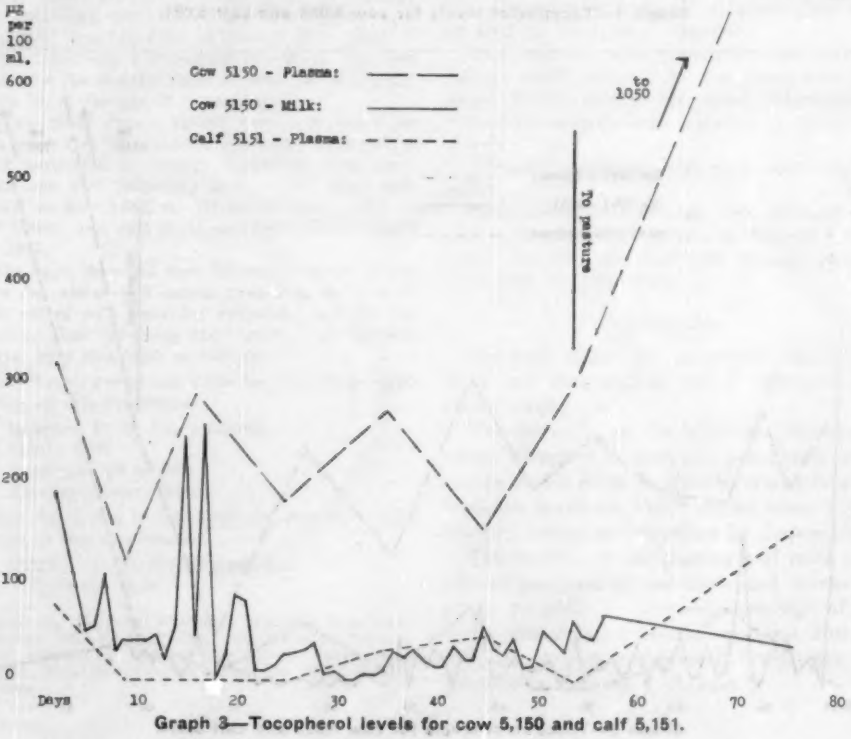
Graph 2—Tocopherol levels for cow 5,093 and calf 5,094.

TABLE 1—Tocopherol Content of the Cows' Milk, and the Cows' and Calves' Blood Plasma (μg./100 ml.)

Date (1953)	Cow 5,068 blood	Cow 5,068 milk (ave.)	Calf 5,091 blood	Cow 5,093 blood	Cow 5,093 milk (ave.)	Calf 5,094 blood	Cow 5,150 blood	Cow 5,150 milk (ave.)	Calf 5,151 blood
3–28	285	75	60	330	420	0			
3–30	405	123			73				
4–3		51	45	225	34	45			
4–6	255	28		315	23				
4–10	225	24	105	210	71	90			
4–18	240	86	120	225	31	22			
4–25	285	34	100	285	24	0	315	185	75
5–2	180	35	0	225	21	0	120	56	0
5–9	285	31	75	350	32	90	285	69	0
5–18	225	43	60	150	27	15	180	56	0
5–28	330	23	120	270	7	75	270	11	30
6–8	120	37	75	60	14	15	150	27	45
6–18	195	33	105	285	31	60	300	35	0
7–7	1755	30	510	1140	12	750	1050	35	150

DISCUSSION

Parrish [4] found that calves, the dams of which received only barn rations, were born with an average serum tocopherol level of 42 μg. per 100 ml. After the consumption of the colostrum, the content of this vitamin rapidly increased in the serum until levels about five times higher were reached the third day. Sharman [5] stated that blood serum figures below 70 μg. of total tocopherol per 100 ml. in calves indicates a low vitamin E status. Blaxter, Watts, and Wood [6] demonstrated that without adequate vitamin E, lesions of muscular dystrophy would develop in the growing calf. Safford, Swingle, and Marsh [7]



Graph 3—Tocopherol levels for cow 5,150 and calf 5,151.

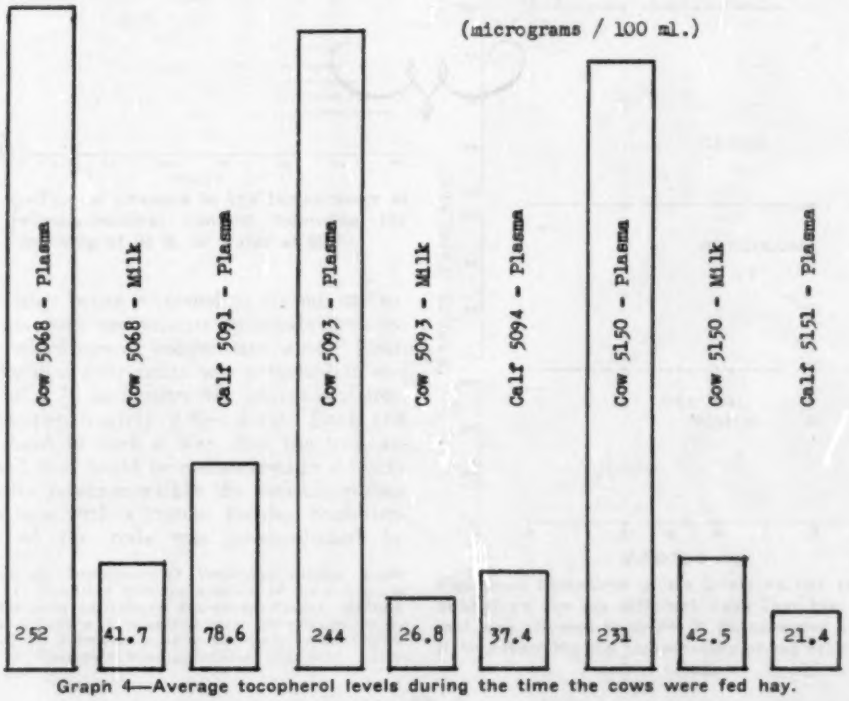
demonstrated that calves, fed a tocopherol-deficient diet and not having tocopherol present in the plasma, developed signs of muscular dystrophy in fourteen to twenty-one days.

Calf 5,094 had an average plasma tocopherol level of 37.4 μg./100 ml. for eighty days with nondetectable amounts on the thirtieth and thirty-sixth days. Calf 5,151 had an average plasma tocopherol level of 21.4 μg./100 ml. for fifty days with nondetectable amounts on the eighth, fifteenth, and twenty-fourth days. Although the plasma tocopherol levels were below 70 μg./100 ml., which is considered a low vitamin E status by Sharman,[5] the calves did not show signs of muscular dystrophy.

The average milk tocopherol levels for cow 5,068 was 41.7 μg./100 ml., cow 5,093 was 26.8 μg./100 ml., and cow 5,150 was 42.5 μg./100 ml. (graph 4). The average milk tocopherol level of 350 samples taken from 38 Montana beef cows from the eighth to thirty-second day of lactation was 124 μg./100 ml.[8] Eighteen milk samples from 18 beef cows whose calves had just died with muscular dystrophy had an average tocopherol level of 104.9 μg./100 ml.[9] Quaife [1] reports a winter milk level in cows to be 80 μg. to 150 μg. of tocopherol per 100 ml. It would appear that the milk tocopherol levels of the 3 cows in our study were lower than normal.

Sharman [5] stated that a blood serum figure below 150 μg. of total tocopherol per 100 ml. in cows indicates a low vitamin E status. Van der Kaay, Teunissen, Emmerie, and van Eeklen [10] found normal cows to have a winter level of 100 to 200 μg./100 ml. of serum tocopherol. The average plasma tocopherol levels of 11 cows on nine Montana ranches at the time their calves died with muscular dystrophy was 521.5 μg./100 ml. The average plasma tocopherol levels of cow 5,068 was 252 μg./100 ml.; of cow 5,093 was 244 μg./100 ml.; and of cow 5,150 was 231 μg./100 ml. (graph 4).

The ratios of the tocopherol levels of the cow's plasma, cow's milk, and the calf's plasma for cow 5,068 was approximately 7:1:2, for cow 5,093 was 9:1:1.4, and for



Graph 4—Average tocopherol levels during the time the cows were fed hay.

cow 5,150 was 11:2:1. There was no correlation between the calf's plasma tocopherol level and the tocopherol level of the milk ingested.

The plasma tocopherol levels of the calves and cows rose to a comparatively high value after being on pasture for 19 days (graphs 1, 2, and 3). The milk tocopherol levels did not rise correspondingly.

SUMMARY

1) Field observations of producing muscular dystrophy in calves transferred to cows whose calves died with the disease could not be repeated with the 3 cows and the 3 calves in this experiment.

2) Comparative milk tocopherol and plasma tocopherol levels were obtained on the cows and calves. A low tocopherol level in 2 of the calves did not result in the occurrence of muscular dystrophy.

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Changes in Temperature of Reticulo-Ruminal Content Following the Drinking of Water

R. D. DILLON, M.S., and R. E. NICHOLS, D.V.M., Ph.D.

Madison, Wisconsin

INSUFFICIENT information is available relative to changes in the temperature of reticulo-ruminal content following the drinking of water, particularly the sequence and magnitude of changes in different areas of the reticulo-rumen.[1,2] Newer instruments now allow synchronous recording and should enable accumulation of more information. A Leeds and Northrup multiple-point recorder (micromax) was used to obtain the data in this report. Attached to the recorder were six nickel-nickel thermohm units, the temperature at

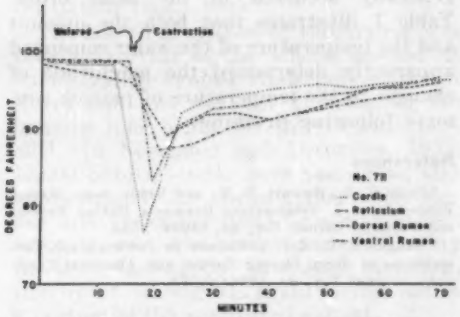

Fig. 1—Typical changes in the temperature of the reticulo-ruminal content following the drinking of 20 lb. of water at 60 F.

each unit being recorded at six-minute intervals with one-minute intervals between the recordings of consecutive units. Each of the first four units was attached to one end of a ¼-inch diameter galvanized iron rod approximately 2 feet long. Each rod was bent in such a way that the unit attached to it could be maintained in a fairly definite position within the reticulo-rumen of a cow with a rumen fistula. Stabilization of the rods was accomplished by clamping the free ends to the fistula plug. The first unit was placed in the cavity of the reticulum, the second a short distance posterior to the cardia, the third deep in the posterior portion of the ventral sac and the fourth in the dorsal sac. The fifth unit was used to record atmospheric temperature and the sixth the rectal temperature. Following attachment of the four rods to the plug, paper towels were compressed into the opening of the plug to close it as effectively as possible.

Figure 1 depicts typical changes following the drinking of 20 lb. of water at 60 F.

Because the time interval between recordings in a particular region seemed to

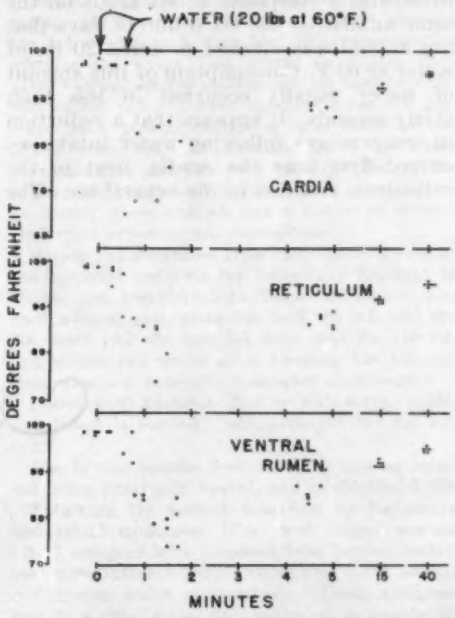

Fig. 2—A composite of six trials on the same animal on the six different days that the animal was allowed to drink 20 lb. of water at 60 F. to determine the temperature of the reticulo-ruminal content.

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The authors wish to express their appreciation for the loan of the instrument used in this study to the Department of Veterinary Science, Purdue University, Lafayette, Ind.

TABLE 1—Magnitude of Changes in the Temperature of Reticulo-Ruminal Contents Following the Drinking of Water

Paunch area	Lowest recording degrees F.	Length of time (minutes) that paunch areas remained below:				
		95 F.	90 F.	80 F.	70 F.	60 F.
		10 LB. OF WATER AT 60 F.				
Cardia	91	13	0	0	0	0
Reticulum	94	15	0	0	0	0
Ventral sac	98	0	0	0	0	0
		20 LB. OF WATER AT 60 F.				
Cardia	76	29	6	1	0	0
Reticulum	85	37	6	0	0	0
Ventral sac	86	52	13	0	0	0
		54 LB. OF WATER AT 50 F.				
Cardia	55	80	40	7	3	1
Reticulum	65	80	32	5	1	0
Ventral sac	70	110	45	13	0	0
		44 LB. OF WATER AT 36 F.				
Cardia	50	70	33	10	3	1
Reticulum	61	70	26	6	2	0
Ventral sac	65	85	43	9	3	0

be too long, the apparatus was altered to record temperatures in only three paunch regions with forty-five seconds between the recordings of the temperatures of the same region and fifteen seconds between the recordings of successive regions. Figure 2 illustrates a composite of six trials on the same animal on the six different days that the animal was allowed to drink 20 lb. of water at 60 F. Consumption of this amount of water usually occurred in less than thirty seconds. It appears that a reduction in temperature following water intake occurred first near the cardia, next in the reticulum, and last in the ventral sac. The greatest reduction occurred near the cardia. Return to prewatering temperatures generally occurred in the same order. Table 1 illustrates that both the amount and the temperature of the water consumed apparently determined the magnitude of changes in the temperature of paunch contents following drinking.

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An Investigation of Antibody Response in Cattle Vaccinated with the Rabbit-Passaged LA Rinderpest Virus in Korea

JUNJI NAKAMURA, D.V.M., Ph.D.; SHIGERU KISHI; JINJI KIUCHI, B.V.M.; ROBERT REISINGER, D.V.M.

Tokyo, Japan

IN AUGUST, 1952, the first field application of a rabbit-passaged strain of the lapinized-avianized rinderpest virus (LA virus) for the vaccination of cattle was made by Reisinger, Mun, and Lee [1] at two villages in the area of Pusan, South Korea. About 180 cattle were inoculated, and no clincial reaction was reported. Pre- and postvaccination serums were collected from a proportionately large number of the vaccinated animals, and antibody response by each animal was investigated by means of complement fixation (c.f.) and virus neutralization (v.n.) tests.

The LA virus method of vaccination was then authorized as the official method of immunization against rinderpest in South Korea and was used for maintaining the immune zone below the thirty-eighth parallel. In November and December, 1952, 15,000 head of cattle were vaccinated and approximately the same number in November and December 1953. No case of adverse reaction has been reported. During the later vaccination, serological investigation by c.f. testing was again carried out in a portion of the vaccinated animals.

In this paper, results of serological investigation in 1952 and 1953 are briefly described.

In 1944, Nakamura and Ishii [2] studied the c.f. antibody response in 85 vaccinated cattle in North Korea, employing their own method for the c.f. test. In this trial, 75 animals (88 %) developed demonstrable c.f. antibody following simultaneous inoculation with the lapinized rinderpest virus and immune serum. In 1950, a similar investigation was carried out during vaccination in Japan.* The 268 cattle which were vaccinated by the lapinized virus-immune serum method were tested, and the c.f. antibody response was confirmed in only 52 animals, or 19 per cent. Many c.f. negative postvaccination serums, however, were proved to be capable of neutralizing the lapinized virus in rabbits, and 19 vaccinated cattle which had failed to develop the c.f. antibody in their postvaccination serum were later found to be solidly immune to challenge by the bovine rinderpest virus.

From the Nippon Institute for Biological Science, Akebonocho, Tachikawa, Tokyo, Japan (Nakamura, Kishi, Kiuchi) and the United Nations Korean Reconstruction Agency (Reisinger).

* Under the cooperation of the Kyushu Branch Laboratory of the Government Experimental Station for Animal Hygiene, Nippon Institute for Biological Science and the Hyogo Prefectural Institute for Rinderpest Serum Production.

MATERIALS AND METHODS

Materials Tested.—For the investigation in 1952, 149 cattle in the rinderpest-free Pusan area were tested. These animals received the LA vaccine on August 17. Prevaccination serum was obtained from each animal immediately preceding vaccination, but postvaccination serum was available on September 6 and 7 from only 91 of these animals, *i.e.*, from twenty to twenty-one days after vaccination.

The investigation in 1953 was begun with 107 cattle. On November 24 these animals received the LA vaccine and were bled for prevaccination samples and again bled fifteen to sixteen days after vaccination for postvaccination samples. Prevaccination samples were obtained from 100 animals, while postvaccination samples could be collected from only 48 animals, in 7 of which prevaccination samples were lacking. Except for a few which had received vaccination about a year previously, these animals had a history of neither rinderpest exposure nor vaccination.

Serum was separated from each blood sample at the National Institute for Veterinary Research in Pusan, and transferred to Tokyo by air. It was used without preheating for both the c.f. and the v.n. tests. All the samples were used for the c.f. test within two weeks after bleeding but the v.n. tests were not generally completed as promptly.

Complement-Fixation Test.—Nakamura, Kishi, and Kiuchi's method [4] was employed for the c.f. test.

The bovine serums were used for testing without being previously heated, and in dilution 1:10.

Following the method described by Nakamura and Ishii,[5] rinderpest (PA) and control normal (NA) antigens were prepared from bovine rinderpest virus-infected lymph nodes and from normal calf lymph nodes, respectively. These antigens, kept in a dried state, were extracted in weakly alkaline medium before use. The final antigen preparations used for the test contained extract from 0.5 Gm. of dried tissue in 100 ml. of saline.

A pool of fresh serums from more than 5 guinea pigs was used for the complement. Starting from

dilution 1:10, it was diluted in 4:5 fold series.

A 2.2 per cent suspension of washed goat blood cells was sensitized with 4 units of immune rabbit hemolysin in an amount not exceeding 5 per cent of the blood cell suspension.

A 0.85 per cent NaCl solution with phosphate buffer pH 7.4 added served as diluent for the reagents and reaction mixtures.

Grades of hemolysis were read in percentage by means of visual comparison to a standard hemolysis series which was daily prepared with the same blood cell suspension as was used in the test, and in such a manner that different percentages of the cells were hemolyzed in water, and then, after addition of sufficient sodium chloride to make the menstruum isotonic, the rest of the cells were supplemented.

The test consisted of two comparable N and P series which were composed similarly except for the antigen used, *i.e.*, NA in N series and PA in P series. Each series consisted of nine test tubes in which 0.2 ml. of complement in serial dilutions was mixed with 0.2 ml. of an aa mixture of serum 1:10 and antigen 1:200. After the fixation period of four hours at 1 to 4 C., to each test tube was added 0.1 ml. of sensitized blood cell suspension; it was left in a water bath at 37 to 38 C. for thirty minutes. The reading was made after standing overnight in a refrigerator.

Specific reaction was estimated by comparison of results in N and P series in a limited area of four to six continuously numbered test tubes. This judging area was chosen at the situation of the series where the average percentage of hemolysis in N series gave the nearest value to 80 per cent. The total percentage of hemolysis in this area was then calculated, respectively, for N and P series, getting values Hn and Hp. The fixation titer (f.t.) was obtained from the following formula, and represented the percentage of blood cells which remained undestroyed as the consequence of specific fixation of complement as compared to the total amount of blood cells which were hemolyzed in N series.

$$\text{f.t. (or 80\% f.t.)} = \frac{\text{Hn} - \text{Hp}}{\text{Hn}} \times 100$$

Some examples illustrated in table 1 may serve for an easier understanding.

The standard for judging as to positive or negative reaction was as follows:

f.t.	*judgment*
0 to 10	Negative
11 to 20	Doubtful
21 or more	Positive

Virus Neutralization Test.—LA virus and embryonating chicken eggs were employed for the demonstration of v.n. antibody.

Since the lapinized rinderpest virus was developed, this virus, in rabbits, has been routinely used for the demonstration of v.n. antibody in either bovine or rabbit serums. Rabbits are still very costly, however, in an experiment such as the v.n. test in which a great number of animals are required and, accordingly, experiments have tended to be limited to a small scale and to special experimental purposes. In this study, LA virus and embryonating eggs were used as a substitute for lapinized virus and rabbits for the first time. The success of this trial, as seen in the subsequent part of this paper, has brought a great advance in eliminating the inconvenience as regards experimental animals.

The LA virus at a passage number between A-E_{70} and A-E_{80} was intravenously inoculated in 12- to 14-day-old eggs. After incubation at 37 to 38 C. for five to six days, spleens were collected from a number of surviving embryos and diluted to a 1:500 suspension in broth (pH 7.2 to 7.4). After a light centrifugation, the supernatant was used for the starting virus (dil.: $\frac{1}{5}\ 10^{-2}$), and tenfold serial dilutions were made up to $\frac{1}{5}\ 10^{-6}$.

Serum was diluted 1:5 with broth. When nec-

TABLE 1—Examples of the Calculation of Fixation Titer

Serum	Antigen	Per cent hemolysis at complement dilution $\frac{1}{10} \times (\frac{4}{5})^n$ n: 10	9	8	7	6	5	4	3	2	Total % hemolysis in judging area	Fixation titer
Prevaccin.	NA	10	20	50	70	95	100	100	100	100	315	
T-70	PA	10	20	50	70	95	100	100	100	100	315	0
Prevaccin.	NA	10	40	60	90	95	100	100	100	100	485	
T- 9	PA	10	40	60	90	95	100	100	100	100	485	0
Prevaccin.	NA	20	40	70	90	100	100	100	100	100	400	
T-58	PA	20	50	70	90	100	100	100	100	100	410	—3
Postvaccin.	NA	10	20	50	70	90	95	100	100	100	405	
T-71	PA	0	0	0	0	0	5	20	60	90	25	94
Postvaccin.	NA	5	10	30	70	90	100	100	100	100	390	
T-48	PA	0	0	5	10	20	40	80	90	100	155	60
Postvaccin.	NA	10	20	40	70	90	100	100	100	100	400	
T-38	PA	10	20	40	60	90	95	100	100	100	385	4

Per cent hemolysis in judging areas are underlined. NA—normal antigen; PA—pest antigen.

TABLE 2—Results of Complement-Fixation (c.f.) Tests (1952)

Serum	Cattle group	Negative f.t. —21 to 10	Doubtful f.t. 11 to 20	Positive* f.t. 21 to 40	41 to 70	71 to 100	Total
Prevaccination	A	58	2	0	0	0	60
	B	88	2	1	0	0	91
				1	0	0	
	Total	146 (96.7 %)	4 (2.6 %)		1 (0.7 %)		151
Postvaccination	B	27 (29.7 %)	7 (7.7 %)	10	22	25	
					57 (62.6 %)		91

* Positive results (fixation titer [f.t.] over 20) are classified in subgroups according to their f.t. values. Tendency to stronger production of c.f. antibody following stronger attack of the virus was discussed by Nakamura and Ishii.[2]

essary, it was filtered through a Berkefeld V filter.

Routinely, 0.5 ml. of each virus dilution was mixed with an equal amount of 1:5 serum. In the control, broth was substituted for serum. The mixture was placed in a refrigerator at 1 to 5 C. for one hour.

In the mixture, dilution of serum was increased from 1:5 to 1:10, and dilution of virus from $\frac{1}{5}10^{-3}$ to $10^{-(x+1)}$.

The infectivity of the mixture was tested by intravenous inoculation of 13- to 14-day-old eggs, ordinarily using seven eggs for each mixture. After incubation at 37 to 38 C. for five to six days, eggs were opened and spleens collected from surviving embryos by groups. Infection of each embryo group was diagnosed from the results of demonstration of rinderpest c.f. antigen in the pooled spleens. The c.f. method here employed was a special one which had been introduced by Nakamura and Kishi[6] for the test with very small quantities of materials and is routinely called "the miniature postdilution method." In this method, calculation of fixation titer and judgment of results are made similarly to the method of Nakamura, Kishi, and Kiuchi as mentioned previously. Characteristic lesions in infected embryos, i.e., enlargement and reddening of the spleen, permitted a decision when the c.f. result was doubtful.

The grade of virus neutralization was calculated as the log difference between maximal infective dilutions of the virus suspensions with and without serum, and represented by a symbol —, +, ++, or +++ as follows:

Log difference	*Grade of v.n.*
0	— (negative)
1	+ (weakly positive)
2	++ (moderately positive)
3 or more	+++ (strongly positive)

RESULTS—SURVEY OF 1952

Complement-Fixation Test.—The c.f. antibody was tested with all the serum samples obtained, i.e., 151 pre- and 91 postvaccination serums. Results are summarized in table 2.

Among 151 prevaccination serums (groups A and B), 146 (96.7 %) were negative, four (2.6 %) doubtful, and only one (0.7 %) was positive with the limit f.t. 21. In contrast to these results, among 92 postvaccination serums (group B), 57 (62.6 %) were positive, seven (7.7 %) doubtful, and 27 (29.7 %) negative. From this result, the c.f. antibody response to the vaccination is evident on the whole. Regarding

TABLE 3—Example of Virus-Neutralization Test (Experiment 6)

Serum (No.)	Serum vaccination	C.f. antibody f.t.	Result of v. n. test* Dilution of virus in mixture 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Grade of v.n.
Broth	.....	....	.....	100	97	97	0	.....
T-70	Pre-	0	100	100	92	88	....	—
T- 9	Pre-	0	100	97	92	63	....	—
T-58	Pre-	—3	99	100	82	93	....	—
T-71	Post-	94	0	5	0	0	....	+++
T-48	Post-	60	32	0	5	0	....	+++
T-44	Post-	7	97	0	0	0	....	+++
T-38	Post-	4	100	100	0	0	....	++
S-10	Post-	0	100	89	100	88	....	—
T-28	Post-	—4	57	0	0	0	....	+++

* Figures represent the titer of the c.f. antigen in chicken embryos inoculated with the virus-serum (virus-broth in control) mixture, 10 or less being negative or "not infected," from 11 to 20 "doubtful," and 21 or more positive or "infected."

C.f.—complement-fixation; v.n.—virus neutralizing; f.t.—fixation titer.

the comparatively low proportion of positive reactions, our previous experiences both in the laboratory and in the field should be remembered. With great reliability, animals can be judged to have acquired immunity when they have developed the specific c.f. antibody in the serum. The reverse, however, is not always true.

TABLE 4—Results of Virus-Neutralization Tests (1952)

f.t. (c.f. reaction)	Prevaccination serum grade of v.n.			Postvaccination serum grade of v.n.				
	−	+ ++ +++	total	−	+	++	+++	total
52-100 (Pos.)	0	0	0	0	0	0	10	10
12-16 (Doubt.)	1	0	1	1	1	0	4	6
10 or less (Neg.)	14	0	14	7	5	9	6	27
Total	15	0	15	8	6	9	20	43

c.f. — complement fixation; v.n. — virus neutralizing; f.t. — fixation titer.

Among the three immune factors produced during a rinderpest infection, *i.e.*, c.f. antibody, v.n. antibody, and true immunity, the c.f. antibody is generally the poorest in development. To reach the demonstrable threshold, the c.f. antibody appears to require a stronger stimulation than do the other two.

Virus Neutralization Test.—The v.n. tests were carried out mainly for the purpose of examining the v.n. antibody content in the c.f. negative and c.f. doubtful postvaccination serums. Thus, all 27 c.f. negative and six of seven c.f. doubtful postvaccination serums were chosen for the test. Ten c.f. positive postvaccination serums and 15 c.f. negative or doubtful prevaccination serums were added as controls. These 58 serums were divided into seven groups, and each group was tested separately.

An example of the test results is shown in table 3.

In this example, the minimum infective dose (m.i.d.) of the control virus was 10^{-6}. Three pools of embryo spleens infected with the virus at a dilution of 10^{-4}, 10^{-5}, and 10^{-6} were all strongly antigenic in the c.f. test (f.t.:100, 97, 97). By any of the three prevaccination serums the virus was not neutralized and it showed the same m.i.d. as the control virus. The two postvaccination serums containing the c.f. antibody of fixation titer 94 and 60 neutralized the virus at 10^{-3} and 10^{-4}, respectively. Among the four c.f. negative postvaccination serums, three were virus neutralizing at 10^{-4} or 10^{-5} dilutions, but the remaining one was not active.

In one of the eight tests the m.i.d. of the control virus represented a dilution as high as 10^{-4} and, consequently, there were left only two dilutions, 10^{-3} and 10^{-4}, for the judgment of virus neutralization by serum samples. Results of this test were not taken, and all the serum samples here employed were retested. Through the remaining seven tests, the m.i.d. of the control virus was relatively uniform. It was 10^{-5} in three tests and 10^{-6} in four tests.

Results of the seven tests are summarized in table 4.

Fifteen prevaccination serums tested (14 c.f. negative and 1 c.f. doubtful) were all negative. As for the postvaccination serums, 10 c.f. positive ones were all strongly virus neutralizing. Among six c.f. doubtful serums, five samples (83 %) were positive, four of them being strongly neutralizing. Also, among 27 c.f. negative serums, 20 samples (74 %) were positive, six of these demonstrating strong neutralizing activity.

TABLE 5—Results of Complement-Fixation (c.f.) Tests (1953)

Serum	Cattle group	Negative f.t. −18 to 10	Doubtful f.t. 11 to 20	Positive* f.t. 21 to 40	41 to 70	71 to 100	Total
Prevaccination	A	58	1	0	0	0	59
	B	35	6	0	0	0	41
				0	0	0	
	Total	93 (93.0 %)	7 (7.0 %)		0 (0 %)		100
Postvaccination	B	11	5	4	10	11	41
	C	2	0	2	0	3	7
				6	10	14	
	Total	13 (27.0 %)	5 (8.7 %)		30 (64.5 %)		48

* Positive results (fixation titer [f.t.] over 20) are classified in subgroups according to their f.t. values. Tendency of stronger production of c.f. antibody following stronger attack of the virus was discussed by Nakamura and Ishii.[3]

Immune Response to Vaccination.—Following vaccination, probably as a consequence of an infection with the LA virus, v.n. and c.f. antibodies were produced in the serum of vaccinated animals. Development of the v.n. antibody, however, was far more regularly demonstrated than that of the c.f. antibody. For an indication of immune response, the v.n. test can be said to be more exact than the c.f. test. The c.f. test, however, has a great advantage in application for mass study in a short time which is generally difficult with the v.n. test, even when the improved method of employing the LA virus and embryonating eggs is adopted. A combination of the two techniques, as illustrated here, appears to be the preferable method.

The results of both tests on the 91 postvaccination serums are summarized in the following groups.

C.f. positive	47
C.f. positive and v.n. positive	10
C.f. doubtful and v.n. positive	5
C.f. negative and v.n. positive	20
Total	82 (90.1 %)
C.f. doubtful and v.n. negative	1
C.f. negative and v.n. negative	7
Total	8 (8.8 %)
C.f. doubtful	1 (1.1 %)

Immune response to vaccination, as indicated by the development of the c.f. antibody or the v.n. antibody or both, was thus proved in 82 (90.1 %) of 91 vaccinated animals.

Following Reisinger, Mun, and Lee,[1] one of the vaccinated cattle, T-52, whose postvaccination serum was negative in the c.f. test (f.t.:3) and moderately positive in the v.n. test in our investigation, was challenged by the bovine virus thirty-eight days after vaccination. This animal was found to be solidly immune.

Results—Survey of 1953

Complement-Fixation Test.—Cattle tested were divided into three groups, *i.e.*, group A of 59 animals furnishing only prevaccination serums, group B of 41 animals furnishing both pre- and postvaccination serums, and group C of 7 animals furnishing only postvaccination serums. In 1953, the test was limited to the demonstration of c.f. antibody. Results obtained are summarized in table 5.

None of the prevaccination serums gave a positive reaction. Among the 41 postvaccination serums, 30 (64.5 %) were positive. This rate of positive reactors approximates results in 1952.

Summary

Antibody response to field vaccination with the LA rinderpest virus was investigated in 91 cattle in 1952 and in 41 cattle in 1953.

In 1952, tests for both complement-fixation (c.f.) and virus-neutralizing (v.n.) antibodies were made with the following results.

1) Development of the c.f. antibody was demonstrated in 62.6 per cent of the vaccinated animals.

2) The v.n. antibody was demonstrated in all of the 10 c.f. positive animals tested, in 5 of the 6 c.f. doubtful animals, and in 20 of the 27 c.f. negative animals.

3) Immune response to the vaccination, as indicated by the development of the c.f. antibody or the v.n. antibody or both, was thus proved in 82 (90.1 %) of 91 vaccinated animals.

In 1953, the c.f. antibody only was tested with the following result.

1) Development of the c.f. antibody was demonstrated in 64.5 per cent of the vaccinated animals.

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A Preliminary Report on the Identification of *Vibrio Fetus*

J. H. BRYNER, A.B., and A. H. FRANK, D.V.M.

Beltsville, Maryland

Much of the work done on *Vibrio fetus* has been concerned with developing a serological test for the diagnosis of vibriosis; however, reports of studies on the serological relationships of strains have been somewhat contradictory.

In 1919, Smith and Taylor [13] reported that Vibrio isolated from aborted bovine fetuses were alike serologically. Later Plastridge, Williams, Easterbrooks, Walker, and Beccia [9] stated that *V. fetus* cultures of bovine origin, unlike those of ovine origin, belong to one serological type. Working with Vibrio of ovine origin, Blakemore and Gledhill [2] reported distinct serological types. Similar findings were reported by Marsh and Firehammer [8] working with both ovine and bovine cultures. Serological differences in Vibrio of bovine origin have been noted by Manthei,[7] Robertstad,[12] and Price, Poelma, and Faber.[11] Also, Levi [6] reported that strains isolated from ovine origin were serologically heterogeneous.

In the diagnosis of vibriosis, major emphasis has been placed on serological procedures to the neglect of identifying and classifying the causative organisms by biochemical means. Bergey [1] does not give positive cultural or biochemical tests for the identification of *V. fetus*. Price [10] reports that Vibrio isolated from aborted fetuses reduce nitrates to nitrites, but do not produce hydrogen sulphide in lead acetate medium.

The following is a report on the study of biochemical and serological relationships of Vibrio isolated from the reproductive tract of cattle and from aborted fetuses.

Materials and Procedures

Vibrio Cultures.—A total of 164 cultures of Vibrio were tested. These cultures were isolated from aborted fetuses, vaginal mucus from cows, semen and preputial samples from bulls, and the reproductive organs from slaughtered cattle. Stock cultures of these isolates were carried in Bacto-thiol medium (thiol)* as recommended by Huddleson [5] and were transferred to fresh mediums about every five weeks. Fresh thiol cultures were used for inoculation of test mediums. Controls were conducted on all test procedures.

Catalase Test.—A modification of the catalase test described by Blom and Christensen [3] was used to determine the catalase activity of Vibrio. A three-day growth of Vibrio in thiol medium was used for the test. The amount of growth was first recorded, then a volume of 3 per cent of hydrogen peroxide (prepared by adding 1 part of commercial 30 per cent H_2O_2 to 9 parts of distilled H_2O) equal to the amount of medium was added to the culture. The cotton plug was replaced by a special stopper provided with a capillary tube. The culture was immediately inverted and the level of the contents marked with a wax pencil. Further mixing was obtained by inverting the tube a few times. The inverted tube was placed in a wire basket over the sink and allowed to stand for twenty minutes. The volume of liquid displaced by gas was measured in millimeters and recorded as the catalase number. Cultures displacing 5 mm. or less of liquid were considered catalase-negative; whereas, those displacing more than this amount were considered catalase-positive.

Reduction of Nitrates.—The culture medium used to test for reduction of nitrates to nitrites consisted of peptone 10 Gm., sodium chloride 5 Gm., beef extract 3 Gm., sodium nitrate 1 Gm., and distilled water to make 1,000 ml., adjusted to pH 7.2 before sterilization. The tubes of nitrate broth were inoculated with 0.5 ml. of concentrated Vibrio growth from the top of thiol medium and were incubated the same as described for the catalase test. Standard reagents, sulphanilic acid and dimethyl-alpha-naphthylamine, were used for the detection of nitrate reduction. A pink to red color was recorded as a positive test for nitrites.

Production of Hydrogen Sulphide.—The culture medium for this test was nutrient broth consisting of peptone 10 Gm., sodium chloride 5 Gm., and beef infusion to make 1,000 ml. Approximately 8 ml. of this medium was distributed in tubes, each of which was inoculated with 0.5 ml. of concentrated three-day growth of Vibrio from thiol. A strip of filter paper impregnated with lead acetate was then inserted into each tube and held in place by the cotton plug. These cultures were incubated in an atmosphere of 10 per cent carbon dioxide at 37.5 C. for twenty-four hours and then examined for reactions on the lead acetate strips. An appreciable darkening of these strips in twenty-four hours was considered a positive hydrogen sulphide reaction; whereas, only a very slight trace or no darkening was considered as being negative.

Deep Stab Growth Test.—The culture medium for this test consisted of thiol with a final concentration of 0.5 per cent agar. A loop-full of three-day-old Vibrio growth from thiol medium was inoculated by deep stab procedure with a platinum loop after which incubation was carried out in an atmosphere of 10 per cent carbon diox-

From the Animal Disease and Parasite Research Branch, U.S.D.A., Agricultural Research Service, Beltsville, Md.

* Bacto-thiol medium (B307) Difco Laboratories, Philadelphia, Pa.

ide at 37.5 C. for three days. Certain types of Vibrio grew along the line of stab in the semisolid medium as well as on top; whereas, other types grew only on or near the surface.

TABLE 1—Vibrio from Aborted Fetuses

Cultures No. and source	Catalase production	Nitrate reduction	Hydrogen sulphide production	Growth in deep stab
27 aborted fetuses	+	+	−	−

Serological Test.—A comparison was made of the antigenic characteristics of catalase-positive and catalase-negative Vibrio. Twenty-five rabbits were hyperimmunized with the catalase-positive type and 12 with the catalase-negative type. Each rabbit was inoculated with a different strain. The antiserum from each rabbit was tested for agglutinability with homologous and heterologous antigens

RESULTS

It was noticed early in 1952 that *V. fetus* grown in thiol produced catalase which could be detected by pouring hydrogen peroxide into the culture tubes and allowing a few seconds for gas bubbles to form. Since then a test has been developed to differentiate Vibrio isolated from cattle into two types; namely, catalase-positive and catalase-negative. All Vibrio cultures isolated from aborted fetuses produced 30 mm. or more of gas with an average of 80 mm. (table 1). Also, many isolations of Vibrio obtained from bovine reproductive tracts, (vaginal mucus from cows, semen and preputial samples from bulls) which were indistinguishable from those isolated from aborted fetuses culturally, biochemically, and morphologically by microscopic methods, produced similar quantities of gas (table 2). In contrast, other cultures of

TABLE 2—Vibrio from the Bovine Reproductive Tract—Catalase Positive

Cultures No. and source	Catalase production	Nitrate reduction	Hydrogen sulphide production	Growth in deep stab
64 from vaginal mucus	+	+	−	−
4 from female reproductive tract postmortem	+	+	−	−
21 from bull semen	+	+	−	−
2 from bull prepuce	+	+	−	−
1 from bull prepuce postmortem	+	+	−	−

Vibrio isolated from the reproductive tracts of cows and bulls either failed to produce any reaction to catalase or never more than 5 mm. of gas (table 3). The growth characteristics on blood agar medium and cellular morphology of catalase-positive and catalase-negative Vibrio types, however, were similar.

Vibrio from aborted fetuses reduced nitrates to nitrites, failed to produce hydrogen sulphide, and grew only on or near the surface of deep stab cultures. These properties were identical to the catalase-positive Vibrio isolated from the reproductive tracts of cows and bulls. Catalase-negative Vibrio isolated from vaginal mucus, semen

TABLE 3—Vibrio from the Bovine Reproductive Tract—Catalase Negative

Cultures No. and source	Catalase production	Nitrate reduction	Hydrogen sulphide production	Growth in deep stab
12 from vaginal mucus	−	+	+	+
23 from bull semen	−	+	+	+
9 from bull prepuce	−	+	+	+
1 from prepuce postmortem	−	+	+	+

and preputial samples reduced nitrates to nitrites, produced large quantities of hydrogen sulphide, and grew well in deep stabs of semisolid thiol as well as on the surface.

All antiserums from the hyperimmunized rabbits showed high agglutinin titers when tested with their homologous antigens. In no case, however, did antigens from strains of the catalase-negative type cross agglutinate with serum from rabbits hyperimmunized with strains of the catalase-positive type. Antigens made from catalase-positive vibrios showed very little or no agglutination with serum from rabbits hyperimmunized with catalase-negative Vibrio. Also, two distinct groups were found within the catalase-negative type on the basis of the serological tests. To date, only limited attempts to classify catalase-positive Vibrio into serological groups have been made.

DISCUSSION

All of the Vibrio isolated from aborted fetuses were catalase-positive and considered to be *V. fetus* on the basis of their source, pathogenicity, morphology, and

growth characteristics. The catalase-positive Vibrio isolated from bovine reproductive tracts were also considered to be *V. fetus* on the basis of their similarity to Vibrio isolated from fetuses and their association with infertility. In all instances, the origin of catalase-positive Vibrio was cows that had a record of being bred or from bulls that had been used for natural service. As reported by Frank and Bryner,[4] most of the infected cows returned to service and infected bulls had a low conception rate. Catalase-positive Vibrio have not been isolated from virgin heifers or virgin bulls.

Catalase-negative Vibrio were not considered to be *V. fetus* since no isolations of this type were made from aborted fetuses.

The occurrence of the catalase-negative type was very confusing because it was occasionally found in conjunction with the catalase-positive type in herds with a history of infertility. In several instances, both types were isolated simultaneously from the same individual. Work in progress, however, indicates that the catalase-negative Vibrio does not cause infertility. This organism was isolated from 2 bulls with conception rates of 1.2 services per pregnancy. Also, it was isolated from 3 cows bred to a bull with an average of 1.2 services per conception. In addition, catalase-negative Vibrio were isolated from 8 virgin bulls which indicates that this type may be contracted from environmental surroundings and its spread in males is not dependent on sexual contact.

Our findings indicated that catalase-positive Vibrio are most frequently spread by natural breeding; whereas, this is not necessarily true in the spread of catalase-negative Vibrio. They also indicate that we are dealing with different species of Vibrio or different strains of *V. fetus* that attack the genital tract of cattle.

SUMMARY

A total of 164 cultures of Vibrio isolated from aborted fetuses, vaginal mucus of cows, semen and preputial washings from bulls were tested for metabolic by-products and growth characteristics in differential culture mediums. There appeared to be two distinct types of Vibrio on the basis of the catalase test: (1) the catalase-positive Vibrio which were thought to be true *Vibrio fetus;* (2) the catalase-negative Vibrio which were not incriminated as causing abortion and infertility in cattle. Both types of Vibrio reduced nitrates to nitrites. The catalase-positive Vibrio did not produce hydrogen sulphide nor grow in deep stab cultures; whereas, catalase-negative Vibrio produced large quantities of hydrogen sulphide and grew in deep stabs. Serological tests indicated that there was little or no relation between the catalase-negative and the catalase-positive types.

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Physiological Effects of Insufflation of the Stomach of Sheep

R. W. DOUGHERTY, D.V.M., M.S.; COURTNEY D. MEREDITH, B.V.Sc.; ROBERT B. BARRETT, B.A.

Ithaca, New York

MUCH of the attention in past and contemporary studies of bloat has been directed toward the etiology and the postmortem changes. Very little effort has been directed toward the physiological changes which occur during acute tympany of the ruminant stomach.

Some studies [1] have shown that gas absorption is rapid and effective from the ruminant stomach. Other volatile and nonvolatile substances have been found [2-4] to be absorbed especially from the rumen and reticulum. Increased gas pressure as well as physical changes in the ingesta have been shown [5] to influence the rate of absorption of certain gases. Changes in the hydrogen ion concentration of the ingesta are known [6] to influence the rate of absorption of certain volatile acids.

A review of the available literature indicates that death from acute bloat is considered to be due to mechanical interference with respiration and circulation. No objective methods have been used to substantiate these subjective observations. The studies herein reported are an attempt to throw some light on the cause of death in bloat; it is hoped that these results will aid in developing a working hypothesis for the etiology of bloat.

A preliminary report was given by Meredith and Barrett [7] at the Biennial Conference on Bloat at Chicago in the fall of 1953. The present report combines the above findings with more extensive work done since that time and includes the effects of insufflation on:

a) arterial blood pressure and arterial blood gases
b) jugular, cerebrospinal fluid, and intrapleural pressures
c) pulmonary circulation time and blood gas concentration
d) arteriovenous differences (blood gases)
e) oxygen utilization.

ARTERIAL BLOOD PRESSURE AND ARTERIAL BLOOD GASES

In this series of experiments, western range sheep possessing permanent ruminal fistulas and in good health were employed. The sheep were restrained in stocks and the gas was insufflated directly into the rumen through the cannula fistula. Gases used for insufflation included oxygen, carbon dioxide, nitrogen, and a mixture of meth-

From the Department of Physiology, New York State Veterinary College, Cornell University, Ithaca.

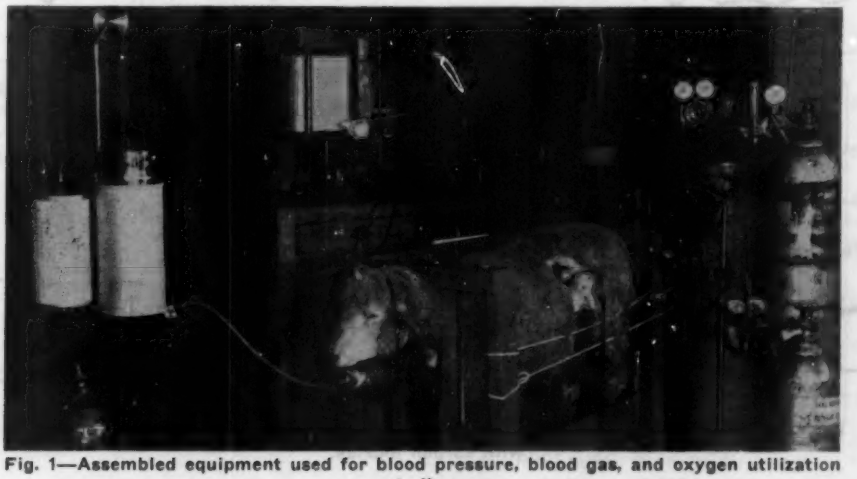


Fig. 1—Assembled equipment used for blood pressure, blood gas, and oxygen utilization studies.

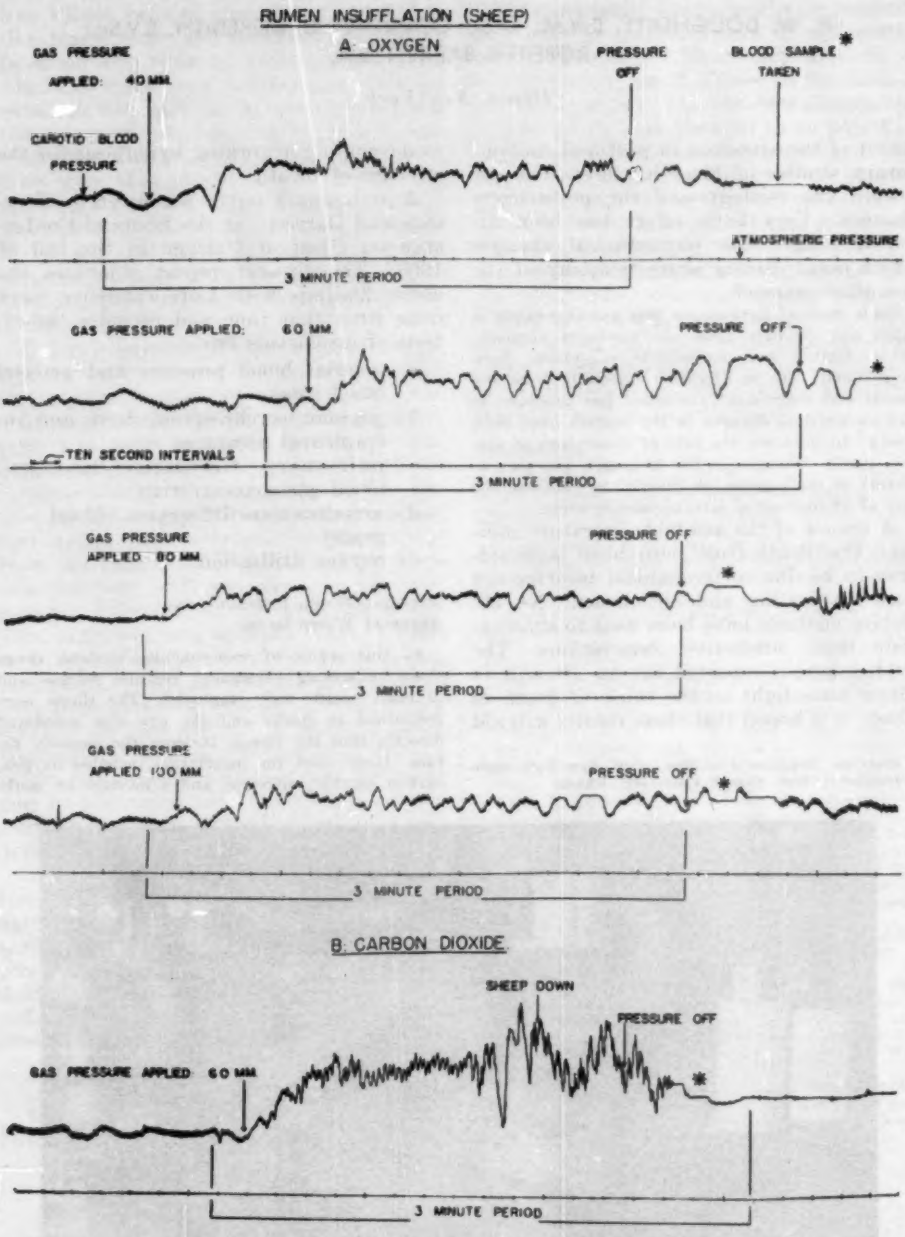


Fig. 2—Effect on blood pressure of oxygen at varying intraruminal pressures as compared with carbon dioxide.

ane and carbon dioxide (40 % methane:60 % carbon dioxide). An aneroid manometer in the system connecting the gas pressure regulator to the rumen cannula made it possible to control the intraruminal pressures fairly accurately. The assembled equipment used in these experiments is illustrated in figure 1.

For recording the arterial blood pressure and obtaining blood samples for analysis, the left carotid artery was cannulated and pressure recorded on a continuous-feed kymograph through a citrate-filled tube and a mercury manometer with an ink-writing point. To ascertain whether the elimination of one carotid artery from the circulation affected the significance of the results obtained, 1 sheep was fitted with a T-shaped cannula thus allowing patency of the carotid to be uninterrupted. No noticeable difference was recorded. Where the ordinary cannula was employed, sufficient blood was withdrawn immediately prior to the taking of the blood sample to remove the theoretical "static" column of blood in the occluded carotid and to endeavor to ensure that the sample was representative of the circulating arterial blood. A three-way stopcock attached to the cannula facilitated this procedure. Blood samples were originally collected in greased syringes under mineral oil, as recommended by Peters and Van Slyke,[4] and coagulation was prevented with heparin. Later the modified technique as described by J. Victor Monke[5] was adopted but heparin was used instead of potassium oxalate. All samples were analyzed in the Van Slyke manometric apparatus for total oxygen and carbon dioxide.

A sheep was subjected to intraruminal pressures of 40, 60, 80, and 100 mm. of mercury using oxygen as the insufflating gas. In each case, the pressure was maintained for three minutes without harmful effect to the animal. When the sheep was insufflated with carbon dioxide, maintaining an intraruminal pressure of 60 mm. of mercury for a period slightly less than three minutes, the animal collapsed and the gas pressure was released. The pressure recordings are illustrated graphically in figure 2. The relative changes in blood pressure and blood gases (carbon dioxide and oxygen), for this part of the experiment, are shown in figure 3.

Figure 4 illustrates the factor of individual variation existing in the susceptibility to pressure, e.g., in individual sheep variable pressures are required to produce changes in blood gas concentration and to produce symptoms of distress. Sheep 1, 2, and 3 were insufflated with the gas mixture (CO_2:CH_4) maintaining an intraruminal pressure of 20 mm. of mercury. Sheep 3 was then subjected to 100 mm. of pressure with the gas mixture and sheep 2, to 30 mm. of pressure with carbon dioxide.

Sheep also vary in susceptibility not only to the pressure required to produce detectable change but also in the time for which elevated pressures can be tolerated. Figure 5 illustrates that in sheep A an intraruminal pressure of 40 mm. produced marked dyspnea and collapse in slightly more than three minutes, whereas sheep B resisted pressure increases up to 80 mm. for just under five minutes before fall in blood pressure, cessation of respiration, and total collapse; in both cases, recovery occurred after approximately thirty seconds following release of intraruminal pressure.

To determine the effects of partial or total obstruction of the posterior vena cava this venous

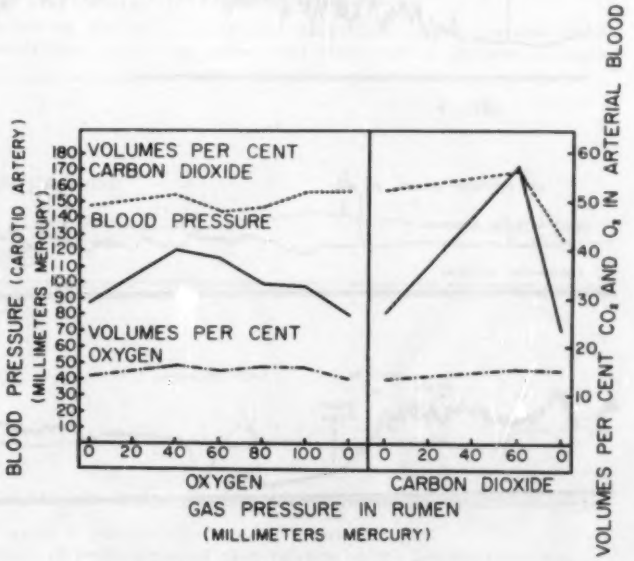
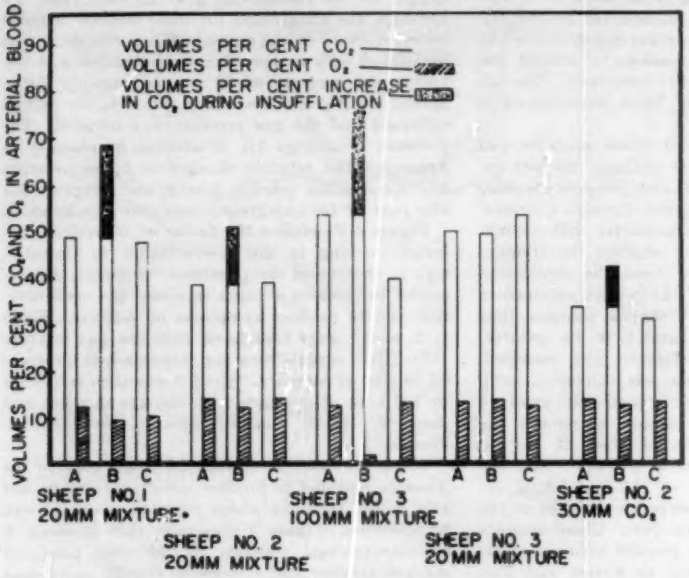


Fig. 3 — Arterial blood pressure and blood gases before, during, and after insufflation with oxygen and carbon dioxide.



A—preinsufflation; B—insufflation; C—postinsufflation; * insufflation pressure and gas.

Fig. 4 — Arterial blood gas variations induced by different intraruminal pressures and gases.

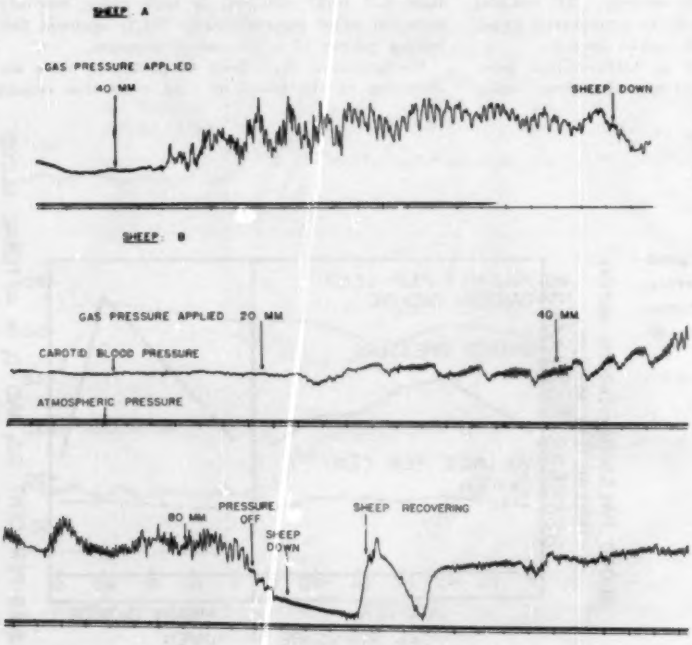


Fig. 5 — Blood pressure recordings illustrating individual variation to rumen insufflation. Time intervals, 10 seconds.

return was ligated temporarily in an anesthetized dog. Total and temporary occlusion of this vessel had only a transient and slight effect on carotid blood pressure. The results are shown in figure 6.

DISCUSSION

In the early work when single arterial blood samples were taken before and during insufflation, the results were consistent, CO_2 was increased and O_2 decreased during insufflation. This work was done during the summer of 1953. Later, in the winter of 1953-1954, during the work on cardiac output (pulmonary circulation time), a marked difference was observed in the color of the consecutive blood samples that had been taken continuously during insufflation. The color of the arterial (carotid) blood varied from bright red to purple. The variation seemed to be correlated with eructation. The changes in color were rapid.

It is possible to develop a number of hypotheses as to the difference in the results obtained using the two different blood-sampling methods. For example, the fluctuations occurred every few seconds. The single samples may have been taken at the same time in each cyclic fluctuation, although this seems improbable. The other possible explanation is that the single samples were taken in summer when the sheep may have had more fluid ingesta, thus influencing both gas absorption and eructation. This explanation also seems to be inadequate. Since the last continuous sampling experiment was done in late June, with results similar to the winter experiments, it is felt that results showing pronounced fluctuations in blood gases corresponding with eructations are more representative.

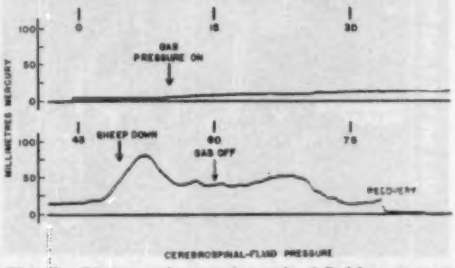
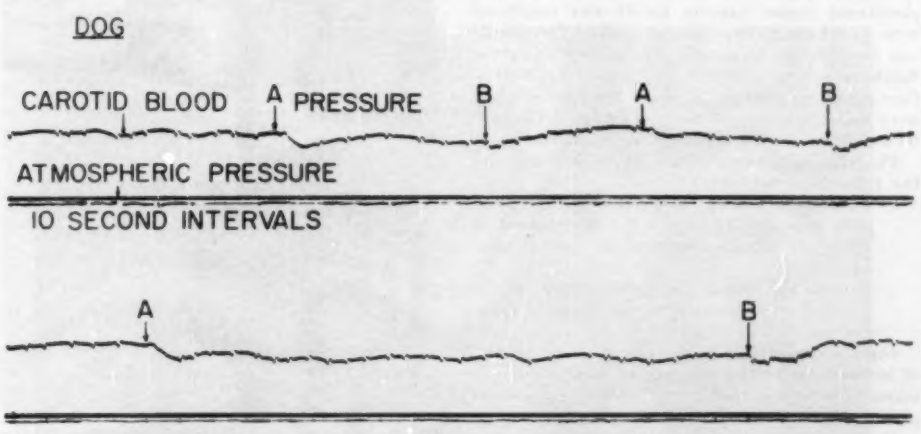


Fig. 7—Changes in cerebrospinal-fluid pressure during rumen insufflation.

The blood pressure rise shown in the short experiments (3 minutes) certainly may not be comparable to the blood pressure changes in the terminal stages of severe bloat. Since the bloating time is variable and since so little is known of the intraruminal pressures occurring in severe bloat, it is difficult to predict the pressures existing in the critical stages of acute tympany.

JUGULAR, CEREBROSPINAL FLUID, AND INTRAPLEURAL PRESSURES

A clinical observation lead the present authors to believe that there was an increase in jugular



A—temporary ligation of posterior vena cava; B—ligature released.

Fig. 6—Effect on blood pressure of posterior vena cava ligation in the anesthetized dog.

Fig. 8—Changes in intrapleural pressure during rumen insufflation.

blood pressure during insufflation of the rumen. Therefore, experiments were performed in which both jugular blood pressure and cerebrospinal fluid pressure were recorded under conditions of rumen insufflation.

Since the pressure in the intracranial veins varies with the cerebrospinal fluid pressure, the latter was determined.

As pointed out by Best and Taylor,[10] "a fall in arterial pressure or a rise in cerebral venous pressure will tend to slow the blood flow through the brain." Such a condition affecting the blood flow through the brain could be a factor leading to the production of tissue anoxia in that organ. The adult cranial cavity and vertebral canal form a rigid, nondilatable structure so that increased oxygenation of the brain in time of distress is possible only through increase in the velocity of the blood flow.

The left jugular vein of a sheep possessing a permanent rumen cannula fistula was punctured with an 18-gauge hypodermic needle. The needle was attached via a polyvinyl tube directly to the Sanborn electromanometer (Model 121B-100). Continuous recordings of blood pressure changes were made through electronic coupling of the electromanometer to the Sanborn viso-cardiette.

The jugular blood pressure was recorded under the following conditions:

1) before and during insufflation of the rumen with gas mixture (CO_2:CH_4) maintained at an intraruminal pressure of 60 mm. of mercury;
2) before and during manual occlusion of the jugular vein proximal to the point of penetration.

Jugular pressures rose sharply to over 100 mm. of mercury under the influence of insufflation, but manual occlusion failed to produce pressures greater than 34 mm. of mercury.

In the other section of this experiment, using the same sheep, recordings of cerebrospinal fluid pressure were made with the apparatus described above after the vertebral canal had been punctured between the third and fourth lumbar vertebrae. The spinal puncture was made under local anesthesia (2 % procaine).

Recordings of cerebrospinal fluid pressure were made before and during insufflation of the rumen with the gas mixture used previously and the results are illustrated in figure 7.

Intrapleural pressures, before and during insufflation, were determined by a direct method. An Ochsner trocar was connected with rubber tubing to a bromoform manometer, the manometer rider arm writing on a kymograph. Several small holes were bored in the cannula of the trocar near its tip. Local anesthesia was used in the surgical area and, with the sheep in the standing position, the trocar was inserted into the left pleural cavity. A purse string suture held the cannula in place between the eighth and ninth ribs. As insufflation was induced, fluctuations in intrapleural pressures increased as shown in figure 8. The greatest posi-

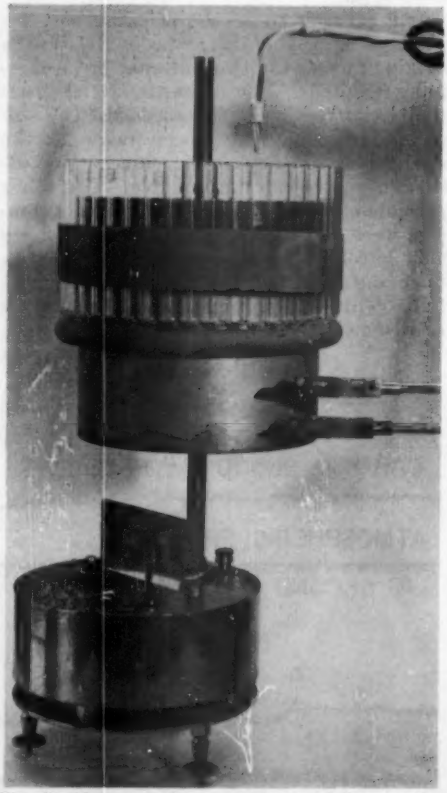


Fig. 9—Kymograph assembly used for continuous sampling of arterial blood.

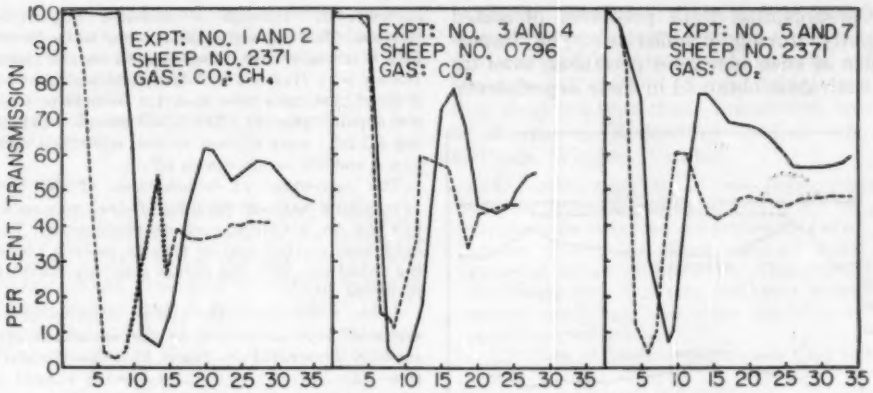

PER CENT TRANSMISSION
EXPT: NO. 1 AND 2
SHEEP NO. 2371
GAS: CO_2: CH_4
EXPT: NO. 3 AND 4
SHEEP NO. 0796
GAS: CO_2
EXPT: NO. 5 AND 7
SHEEP NO. 2371
GAS: CO_2
Time in seconds; circulation time during insufflation (·····); normal circulation time (——).

Fig. 10—Circulation times determined by Hamilton's dye injection method in normal and insufflated sheep.

tive pressure occurred just before eructation and was usually no more than 40 mm. of mercury.

To determine if closure of the glottis was responsible for the transient rise in intrapleural pressure, the experiment was repeated, the only difference being that a tracheal tube was inserted in the cranial part of the trachea. With the respiratory tract open, the intrapleural pressure showed only a transient rise of approximately 9 mm. of mercury above atmospheric pressure. This indicates that closure of the glottis was involved in the initial part of the eructation mechanism.

DISCUSSION

Intrapleural pressures as determined by direct methods remained fairly constant except for an instant before eructation. During this period, the glottis was apparently closed. The highest intrapleural pressure recorded during severe insufflation was 40 mm. of mercury. Since intrapleural pressures remained fairly constant, the explanation for the rapid rise in jugular pressure becomes somewhat obscure. The rise in intrapleural pressure was so transient that its influence on venous return in the anterior venae cavae could have been only slight. These circulatory studies simply mark the beginning of work that should be continued and should eventually be helpful in making the ultimate studies on the circulatory events occurring in acute tympany.

Fig. 11—Assembled equipment used for continuous arterial blood sampling.

Cerebrospinal fluid pressures increased rapidly during insufflation. The importance of such increased pressures, even up to the values obtained in these experiments,

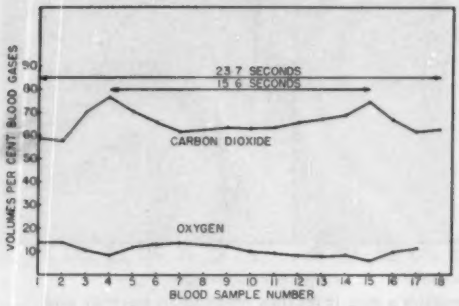


Fig. 12—Continuous determination of total arterial blood gases in the sheep.

may be a contributory factor in some of the central nervous system disturbances seen during severe insufflation.

Pulmonary Circulation Time and Blood Gas Concentration

Since much has been written on bloat and the subsequent effects of increased intraruminal and intra-abdominal pressures on blood circulation and respiration, this series of experiments was designed to show circulatory and blood gas changes under *in vivo* conditions.

The pulmonary circulation time of sheep has been determined using the dye-injection method

TABLE 1—Results Obtained in Determining Pulmonary Circulation Time by Hamilton's Dye Injection Method

Exper.	Date	Insufflating gas and pressure	Remarks
1†	?	No gas	
2†	3–18–54	60 mm. gas mixture	5 sec. (approx.) decrease in circ. time
3*	3–24–54	No gas	
4*	4–8–54	60 mm. CO_2	1½ sec. (approx.) decrease in circ. time
5†	4–13–54	No gas	
7†	4–24–54	60 mm. CO_2	1½ sec. (approx.) decrease in circ. time

* Sheep 0796; † sheep 2371.

of Hamilton *et al.* [11,12] Four milliliters of a 1 per cent solution of Evans blue dye (W761-1)* were injected into the left jugular vein of the experimental animal. Continuous blood samples were collected from the right carotid artery, previously exteriorized,[13] through a siliconed polyethylene cannula. This allowed arterial blood to be directed into a series of test tubes located on the circumference of a Harvard kymograph revolving at such a speed that each tube took 1.2 seconds to pass a given point (fig. 9). The blood samples (averaging 5.7 ml.) were allowed to clot, were then centrifuged and the serum drawn off.

The percentage of transmission of the serum (containing varying amounts of dye) was read at 610 mμ on a Coleman spectrophotometer. These data were plotted against time in seconds (following injection) with the curves resulting illustrated in figure 10.

Table 1 delineates the salient features of this series of experiments, while the assembled apparatus is illustrated in figure 11. The circulation time was derived under the following conditions: (1) comparison before and during carbon dioxide insufflation at an intraruminal pressure of 60 mm. using different sheep; (2) comparison before and during insufflation of carbon dioxide and gas mixture (CO_2:CH_4) using the same sheep.

Utilizing the method for collection of continuous arterial blood samples described in the preceding paragraphs, analyses were performed to determine normal fluctuation in total blood CO_2 and O_2.

The blood was collected in receiving tubes to which 3 drops of heparin (10 mg./ml.) and 1 ml. of mineral oil had been added previously. The heparin was used to prevent coagulation and the mineral oil to prevent gaseous interchange between the sample and the atmosphere after the sample had been procured. An undetermined amount of gaseous interchange occurred at the time of discharge from the end of the cannula to the time when the sample was sealed under mineral oil; however, this exchange under the conditions of the experiment would be constant.

Each blood sample was thoroughly mixed and 1 ml. withdrawn to be analyzed for total blood gases in the Van Slyke manometric apparatus.

In the first series, carbon dioxide was insufflated

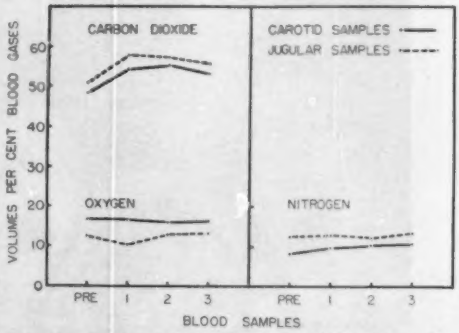


Fig. 13—Arterovenous blood gas differences determined by a method of continuous blood sampling.

* Wm. R. Warner, Division of Warner-Hudnut, Inc., New York.

into the rumen and a pressure of 60 mm. of mercury was maintained for one minute before and during the sampling process. The results of this series are illustrated in figure 12.

In the second series, nitrogen replaced carbon dioxide as the insufflating gas with similar but less dramatic results.

DISCUSSION

According to Best and Taylor,[10] the pulmonary circulation time may be taken as an index of the mean velocity of the pulmonary blood flow and therefore bears a relation to the total volume of blood transversing the lungs in a given time, thus relating circulation time to cardiac output.

The continuous blood sampling and dye techniques make the method valueless in the study of circulatory changes in acute tympany. A method of measuring circulation time without these limitations would be desirable. Regarding cardiac output methods, Wiggers[14] states:

> As regards reliability of these different methods, the following can be said: (1) All involve uncertainties as to length measurements of arteries for velocity calculations, and all introduce empirical values in equations. This introduces the danger that they may not apply under abnormal conditions, even when shown to fit in strictly normal states.
>
> (2) Each of these methods when used by its

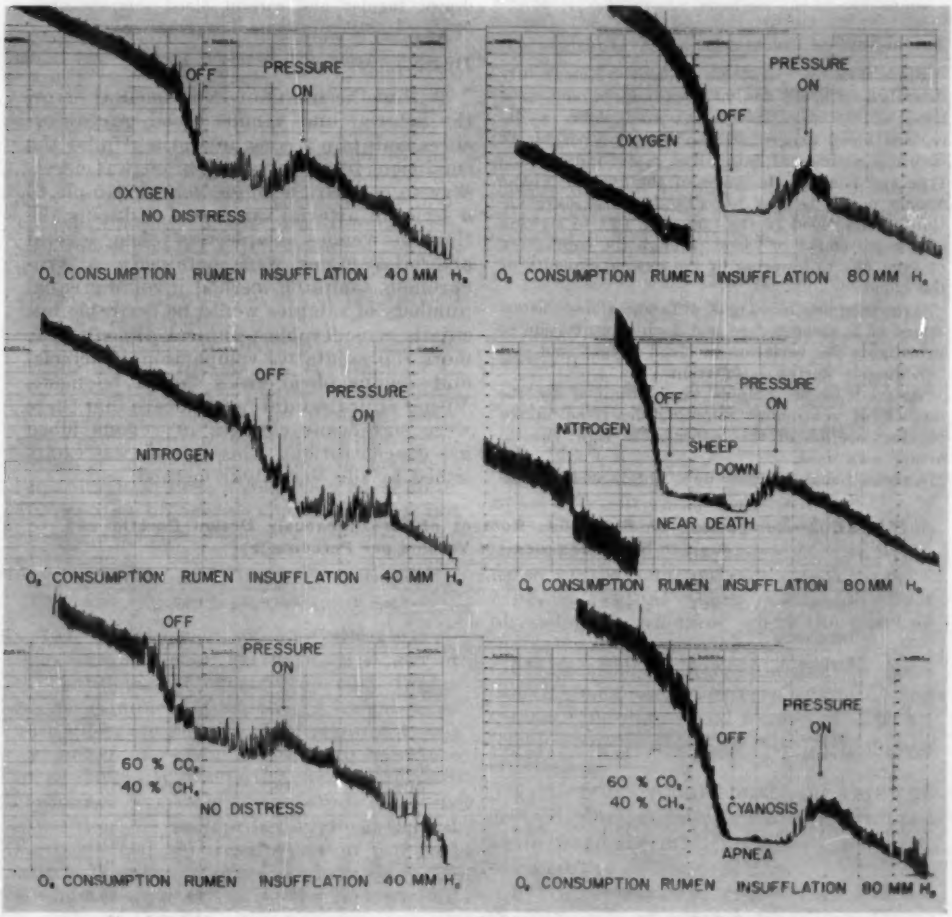


Fig. 14—Oxygen utilization using three insufflating gases under two different pressures.

sponsors can yield results which are in agreement with values for cardiac output determined by gasometric methods; but when the sponsor of one procedure tries that of another, inconsistent checks are reported.

(3) The methods depend on meticulous care in pulse registrations and considerable preliminary training to avoid the many pitfalls in curve analysis. Hence, they can not be recommended except for individuals with adequate laboratory experience.

Finally, it may be noted that recent comparisons with volume flow determined in the aortae of animals by electromagnetic flow meters have shown that, at rest, good (?) correspondence generally occurs with values derived by use of the Broemser and Ranke formula (maximum deviation, 34 per cent). This does not inspire confidence in the methods.

ARTERIOVENOUS DIFFERENCES

Since terminal symptoms of bloat are frequently described as being due to tissue anoxia, and since some of the work herein reported shows a decreased blood oxygen, it was thought advisable to obtain experimental data which might indicate the type and possibly the cause of the anoxia. Tissue anoxia might be due to: (1) defective oxygenation of the blood in the lungs, or (2) to slowing of the movement of blood through the capillaries, or even (3) to lowering of the oxygen capacity of the blood.

Arteriovenous blood gas differences were determined on a sheep before and during insufflation to investigate the variation in blood oxygen and carbon dioxide during insufflation.

Arterial blood samples were obtained by the method of continuous sampling described in the previous section of this paper. The right carotid artery was used.

Venous blood samples were obtained from the left jugular vein using 10-cc. hypodermic syringes as collection receivers. The syringes were prepared by coating the barrel with stopcock grease then flushing the cylinder with a 10 mg./ml. heparin solution. After collection of the blood sample, 1 ml. of mercury was drawn into the syringe to facilitate mixing of the sample with the heparin solution. The syringes were then sealed to prevent gaseous interchange with the atmosphere. This method of collection of blood samples for Van Slyke manometric determinations is described more fully in the literature elsewhere.[8]

Accentuation of a signal marker on the kymograph drum during the time of withdrawal of the venous samples permitted correlation of these samples with the arterial blood samples collected continuously over the period of the experiment. Table 2 illustrated the comparison of simultaneously drawn jugular and carotid blood samples while figure 13 illustrates these data graphically.

DISCUSSION

Within the limits of experimental error, the arterial and venous blood gas differences maintain a constant ratio. Under the conditions of the experiment, it was necessary to compare a single venous sample to a series of arterial samples, *i.e.*, during the time one venous sample was taken, several arterial samples were obtained. A synchronous sampling method involving equal numbers of samples would be desirable but entails considerable technical difficulty. A more representative comparison of arterial and venous blood gases should be made. Visual appraisal did not indicate that there were significant changes in venous blood gas concentrations. Gas volume was established by the Van Slyke method.

TABLE 2—Comparison of Blood Gas Content of Simultaneously Drawn Carotid and Jugular Blood Samples (in Volume per Percentage)

Plot	Jugular	CO_2	O_2	N_2	Carotid	CO_2	O_2	N_2
Pre	Syringe 1 (Prejugular)	51.1	12.5	12.5	Syringe 2 (Precarotid)	48.3	16.7	8.3
1	Syringe 3	57.8	10.4	12.9	Tube 4	55.7	16.4	10.5
					Tube 5	53.0	17.3	9.3
					Tube 6	50.5	17.8	10.3
					Tube 7	57.9	14.8	8.8
					Ave.	54.3	16.6	9.7
2	Syringe 4	57.2	12.8	12.0	Tube 15	66.3	13.6	8.6
					Tube 16	53.6	16.9	11.9
					Tube 17	46.0	17.6	10.7
					Ave.	55.3	16.0	10.4
3	Syringe 5	55.8	13.1	13.4	Tube 24	47.6	18.1	10.1
					Tube 25	48.8	17.2	11.5
					Tube 26	58.4	14.8	10.9
					Tube 27	58.7	15.2	10.0
					Ave.	53.4	16.3	10.6

These values are plotted in figure 13. Series 1 through 3 represent samples taken during insufflation, commencing after 60 mm. Hg. pressure had been maintained for one minute, while the "pre" series represents samples taken before insufflation.

The fraction represented as nitrogen includes all other physiologically inert gases.

Oxygen Utilization

These experiments were designed to determine oxygen utilization by sheep under varying degrees of intraruminal pressure using several different gases for insufflation. Sheep with permanent rumen cannula fistulas were used; the trachea was cannulated surgically, under local and topical anesthesia, the intact respiratory trunk being secured to a Benedict-Roth spirometer by a cuffed Magill tube, making the lungs, connecting tubes, and spirometer into a closed system. The assembled equipment is illustrated in figure 1.

Insufflating gases used were carbon dioxide, oxygen, nitrogen, and the gas mixture (CO_2:CH_4). Various pressures were used ranging from 40 to 80 mm. of mercury. Figure 14 shows oxygen utilization recordings using three insufflating gases under two different pressures, 40 and 80 mm. of mercury, respectively.

Discussion

Because of the acute nature of the experiments, a different sheep was used for each of the trials. Since there is some individual difference in the intraruminal pressure tolerated and in physiological changes produced by the different gases, some variations in the graphs can not be interpreted as being significant.

The decrease in oxygen consumption during severe insufflation corresponds to the arterial blood gas picture. These measurements presented some unusual problems; excitement and struggling during insufflation made it impossible to maintain the animals in even a relatively basal state. The results of these experiments are not exact values and should be accepted only as trends.

General Discussion

This report covers some physiological effects of insufflation. It is not a report of the physiological effects occurring during bloat or induced tympany. Clinical bloat with all of its physiological manifestations has not been reproduced and to be where it is naturally occurring, with adequate equipment available for its study, is quite a problem. It is hoped, therefore, that at least some of the results herein reported will be helpful to other workers more favorably located as far as the incidence of bloat is concerned.

Throughout the work, a wide difference was observed in susceptibility to insufflation. A pressure of 40 mm. of mercury in the rumen might cause great discomfort in one animal, while another would tolerate the same pressure, using the same insufflating gas, in relative comfort. At times, the same animal would vary considerably from day to day in its ability to tolerate insufflation. Although the reasons for these variations are not known, the implications are obvious.

Although complete clarification of the physical events occurring during insufflation has not been achieved in this work, it offers experimental evidence which may provide a basis for additional work on clinical bloat cases. There has been a tendency to put the terminal symptoms on a relatively simple basis. It is doubtful if the circulatory and respiratory changes are as simple as they have been described.

It is impossible to say that the symptoms of acute and severe insufflation are due to mechanical factors only. It would be doubly difficult to blame the symptoms of severe bloat entirely on the impediment of circulation and respiration.

Summary

1) Physiological effects on insufflation of the ruminant stomach were measured and recorded.

2) Sheep with permanent rumen cannula fistulas were insufflated with O_2, N_2, CO_2, and a mixture of CO_2 (60 %) and CH_4 (40 %).

3) Physiological differences occurred when different gases were used.

4) There were marked individual differences in tolerance to the same gas insufflated at the same pressures.

5) Individual sheep varied in their physiological reactions when the gas and the pressures were constant.

6) Increased intraruminal pressure caused sharp rises in arterial (carotid) and venous (jugular) blood pressures. The arterial pressure dropped abruptly during eructation.

7) Cerebrospinal fluid pressure increased during insufflation and dropped again when the intraruminal pressure was released.

8) Intrapleural pressures varied under the influence of labored breathing during insufflation. The only marked variation

was for an instant prior to eructation when the glottis was apparently closed. The transient intrapleural pressure did not exceed 40 mm. of mercury.

9) Insufflation had a pronounced effect on blood gases. The changes were rapid and were influenced by the immediate intraruminal pressures. Even during a short insufflation period, they varied during and after eructation.

10) Short insufflation periods decreased the pulmonary circulation time.

11) Oxygen utilization decreased under the influence of insufflation.

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The Effect of Age and Pregnancy on Ovine Blood Protein Fractions

J. S. DUNLAP, D.V.M., M.S., and W. M. DICKSON, D.V.M., M.S.

Pullman, Washington

BLOOD SERUM protein, globulin, and albumin levels are often used in investigations concerning animal nutrition and animal disease. Before using these values in the appraisal of a nutritive state or a pathological condition, it is important to understand the normal levels and to recognize factors which might affect these levels.

Pregnancy is one of the factors which may alter the blood protein and protein fractions. During this period, the metabolic activities of the ewe are directed toward the support of the fetus and the oncoming lactation period. Howe in 1922 [8] and Howe and Sanderson in 1924 [9] reported little change in the blood proteins of the cow during the gestational period. The investigations of Dieckmann and Wegner [4] revealed a slight but definite decrease in serum protein percentage during pregnancy in women. There was, however, an increase in total protein due to large blood volume changes. Dalgarno and co-workers [3] found evidence of a decrease in the serum globulin of ewes during the latter part of the gestational period; electrophoretic methods were used in this investigation.

It is generally agreed that the serum globulin of young animals is lower than that of adults. Howe [8] found that calves attained adult globulin levels between 3 and 6 months of age when colostrum was fed; the blood globulin increase was slower when colostrum was withheld. Earle [6] verified this finding in foals, kids, lambs, and pigs. Furthermore, Earle found that feeding colostrum resulted in a definite increase of total serum nitrogen within twenty-four hours. McCarthy and McDougall [10] found a rapid increase in serum globulin in lambs which suckled normally; this increase was attributed primarily to *gamma* globulin. These investigators also reported a transient fall in serum albumin after birth; normal adult values returned in about five weeks. Becker and Smith [2] reported that mature sheep had a significantly higher level of total plasma protein than yearlings and that yearlings were significantly higher than lambs.

The purpose of this investigation was to study (1) the effect of pregnancy on blood serum protein fractions; (2) the effect of age as determined by a ten-week period immediately prior to weaning; and (3) the effect of age as determined by ewe-lamb comparisons.

From the Department of Veterinary Pathology and Department of Veterinary Physiology and Pharmacology, State College of Washington, Pullman; scientific paper 1265, project 1056, Washington Agricultural Experiment Stations, Pullman.

MATERIALS AND METHODS

Six crossbred ewes over 1 year of age served as the aged subjects for this experiment. The ewes were bled weekly for twenty-five consecutive weeks. In 5 of the 6 ewes, this included the entire gestational period. Only these 5 ewes were used in analyzing the effects of pregnancy.

The lambs under experimentation were from the above mentioned ewes, bred to the same ram. Each lamb was bled first at 7 weeks of age and weekly thereafter for ten consecutive weeks.

The animals were confined to a small pen, fed hay exclusively, and given iodized salt and water *ad libitum*. Shelter was provided by an open-sided shed with a concrete floor covered with straw. The lambs were not removed from the ewes during the course of the experiment.

The blood samples were withdrawn from the external jugular vein by means of a California-type bleeding needle in the ewes and a 20-gauge needle and syringe in the lambs. Approximately 10 ml. of blood was taken per bleeding for analysis. The blood serum was analyzed separately for total protein, albumin, and globulin using an electrophotometric method based upon that of Fister.[7] This method utilized sodium sulfate in the protein separation and a phenol reagent (Folin-Ciocalteu) to develop the color. The readings were made with a Fisher electrophotometer using filter 650-A.

Environmental temperatures were obtained from a recording thermometer located near the pen.

RESULTS

Pregnancy.—Five ewes were bled weekly during the entire gestational period. Curvilinear regressions were calculated for each fraction of each ewe, using orthogonal polynomials.[1] Graphs were constructed of these regressions on the basis of days prepartum for each bleeding (fig. 1 and 2). The serum globulin concentration underwent a decided decrease during the latter half of pregnancy; this decrease was very significant ($P < .01$) in the linear component of the regression in all cases. In 3 of the 5 ewes, the quadratic component was significant ($P < .05$). Examination of figure 1 revealed that the quadratic signifi-

TABLE 1—Comparison of Mother-Lamb Blood Protein Concentrations*

Animal No.		Blood serum globulin		Blood serum albumin		Blood serum protein	
Mother	Lamb	Mother	Lamb	Mother	Lamb	Mother	Lamb
6	415	3.64	2.72	3.93	3.97	7.39	6.63
27	418	3.98	2.44	3.72	3.88	7.31	6.18
28	417	4.58	2.55	3.38	3.89	7.59	6.24
51	416	4.01	2.48	3.83	3.82	7.58	6.36
52	419	3.85	2.84	3.82	3.87	7.48	6.65
412	420	4.66	3.39	3.28	3.62	7.58	6.92
Mean values		4.12	2.74	3.66	3.84	7.49	6.50
		$t = 8.2883^{**}$		$t = 2.1661$		$t = 8.4918^{**}$	

* Mean values in grams per 100 ml. of serum; ** $P < .01$.

cance was due to the reduction of blood globulin levels at a greater rate during the last part of the gestational period as contrasted with the first part.

In 3 of the 5 ewes studied, the blood albumin concentration was reduced during the first half of the gestational period but nearly recovered to the original levels at parturition. This curve was reflected in the statistical analysis in that these 3 ewes showed a significant quadratic component in the serum albumin.

The total serum protein values showed a steady decrease throughout gestation. This effect probably resulted from the combination of the early depression of serum albumin and the later, and more pronounced, depression of serum globulin values. Thus, as expected, the total serum protein represents an aggregate of two effects.

Age.—The results of the investigation concerning the effect of age can be divided into two parts; (1) The effect exerted by aging during the ten weeks that the lambs were bled, (2) the difference between the blood protein fractions of ewe and lamb.

Analysis of Data from 6 Lambs Bled Weekly from 7 Weeks of Age to 17 Weeks of Age.—These data were analyzed, using orthogonal polynomials, in the same man-

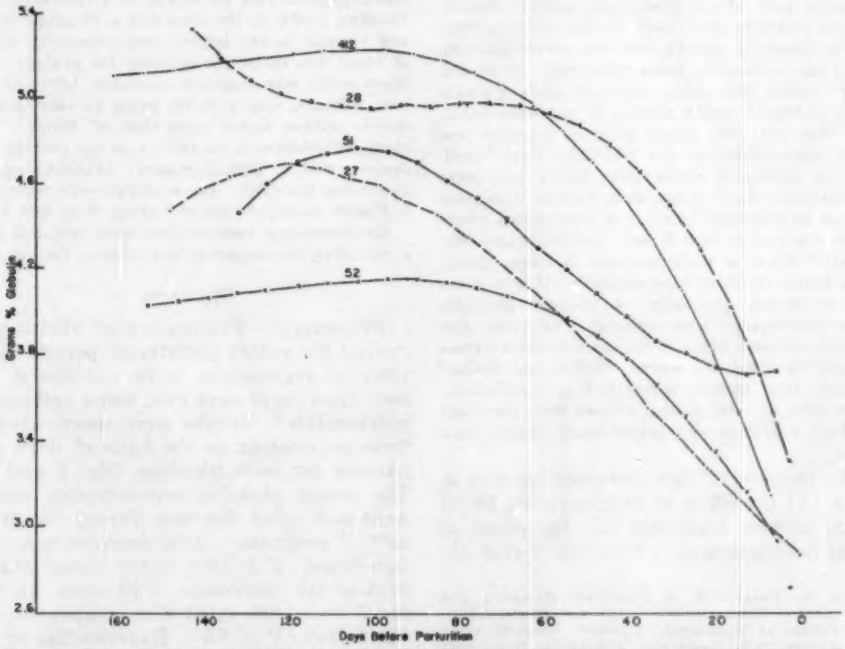

Fig. 1—The effect of gestation on the globulin concentration in the blood serum of 5 ewes.

ner as the pregnancy data. Three of the 6 lambs studied showed a very significant linear increase in serum globulin during the ten-week period. Two of the remaining had a slight but not significant increase. The sixth lamb, 415, had a very erratic serum globulin curve; a definite decrease in serum globulin was observed during the first three weeks, followed by a rise to initial levels at the end of the eighth week and another drop during the last two weeks (fig. 3). There was no significant change in the blood serum albumin or total serum protein of any of the lambs during this ten-week bleeding period.

Analysis of Ewe-Lamb Comparisons.—Methods as given in Snedecor [11] were used for this analysis. The ewes had much higher globulin levels than their offspring; this difference was statistically very significant but could be attributed entirely to the globulin. There was no significant difference in blood serum albumin levels (table 1).

Discussion

With regard to pregnancy, these results parallel the work of Dalgarno [3] and coworkers. The serum globulin of the pregnant ewe decreased markedly during the latter part of the gestational period. This effect may be partially attributed to the production of colostrum by the pregnant ewe, since colostrum is very high in globulin. Partition of the blood serum protein into two components has the advantage of demonstrating exactly where the decrease begins and which component is responsible. If serum protein levels had been used exclusively in this work, the decrease occurring during gestation would have appeared to be a steady linear effect. This was actually a combination effect since serum albumin decreased during the first part of pregnancy, and serum globulin decreased during the latter part. No confident explanation can be offered for the decrease of the serum albumin during the first part of pregnancy. It is possible that during this period of rapid fetal growth, the albumin acts as a nutritive substance; however, this effect has not been reported previously. In an attempt to discover the reason for the changes in blood serum albumin, a survey of environmental temperatures was made during the experimental period. A very significant correlation could

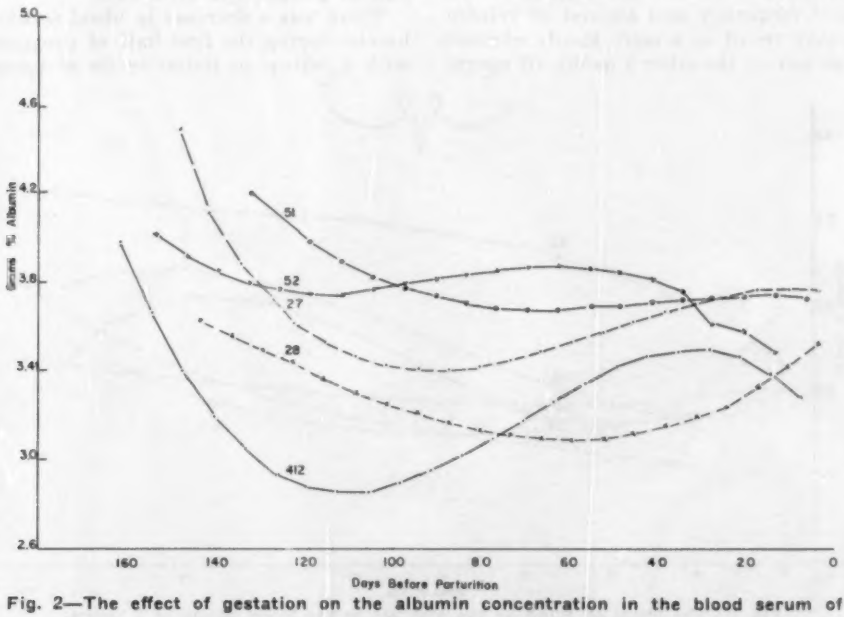

Fig. 2—The effect of gestation on the albumin concentration in the blood serum of 5 ewes.

be demonstrated between serum albumin and either maximum or minimum daily temperatures. Since serum albumin levels were decreasing during November and the first part of December and increasing during the remainder of December and January, it was also considered possible that the amount of light rather than the temperature was a factor. An investigation in which heat and/or light were adjusted revealed no significant effect on blood albumin or globulin by these treatments.[5]

The observations concerning the effect of age on blood serum protein factors substantiate those of other investigators. Young animals have significantly lower serum globulin levels than do older animals. These findings suggest that this rise in globulin can be demonstrated in the relatively short time of ten weeks. The rise may be due, in part, to an increase in antibody globulin due to exposure to subclinical infectious conditions. The erratic results with lamb 415 are interesting in light of the fact that this lamb was born considerably earlier than the other lambs. Since it was the sole member of the young animal population throughout most of its ten-week bleeding period, the chances of infection and reinfection would be diminished. Increased frequency and amount of reinfection may result in a more steady globulin rise as seen in the other 5 lambs. Of course, this is speculative and should be investigated, using additional animals.

Blood volume changes were not determined in this experiment so that no information was obtained concerning total serum globulin or serum albumin. In view of the results of others, blood volume undergoes a definite change during the gestational period.[4] In fact, Dieckmann and Wegner [4] found that whereas serum globulin percentage decreased in late pregnancy the total serum globulin was increased.

This work clearly indicates that partition of blood serum protein into at least two fractions is necessary for concise interpretation of the results. Blood serum protein concentrations are combination effects, and as such do not render a clear picture of the situation since the albumin and globulin may be varying independently.

SUMMARY

1) The blood serum protein, blood serum globulin, and blood serum albumin concentrations of pregnant ewes were determined weekly throughout the gestational period.

There was a very significant decrease in blood serum globulin levels during the last half of gestation in all 5 ewes.

There was a decrease in blood serum albumin during the first half of pregnancy, with a return to initial levels at parturi-

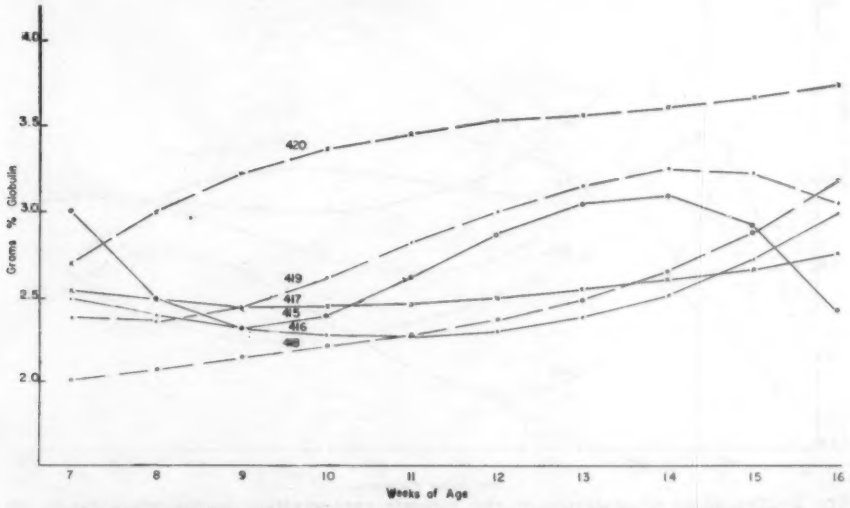

Fig. 3—The effect of aging on the globulin in the blood serum of 6 lambs.

tion. This was very significant in 3 of the 5 ewes studied.

2) The blood serum protein, blood serum globulin, and blood serum albumin levels of 6 weanling lambs were determined weekly from 7 weeks of age to 17 weeks of age.

There was an increase in blood serum globulin in 5 of the 6 lambs studied. In 3 of the 5, the increase was very significant.

There was no significant change in blood albumin.

3) The mean levels for the ewes were compared with those of their lambs.

The ewes averaged 1.38 Gm. more of globulin per 100 ml. of serum than their lambs. This difference was very significant.

The lambs averaged 0.18 Gm. more of albumin per 100 ml. of serum than the mothers. This was not significant.

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Cinefluorographic Studies of the Ruminant Stomach and of Eructation

R. W. DOUGHERTY, D.V.M., M.S., and COURTNEY D. MEREDITH, B.V.Sc.

Ithaca, New York

THE STUDY of the eructation mechanism in ruminants has presented many difficulties. The use of open fistulas has been most productive in studying motility of the ruminant stomach,[1] rumination,[2,3] and digestion [4-6] in both cattle and sheep. Eructation has not lent itself to the most advantageous study through the open fistula for obvious reasons.

The ruminant eructating mechanism has assumed great importance in recent years, since bloat is considered, by most investigators, to be due to faulty eructation. Many of the factors affecting eructation adversely are as yet undetermined.

Recent studies [7,8] have shown the great possibilities of cinefluorography in the study of certain physiological processes in which anatomical structure and action or motility are of major importance.

Considerable work has been done on man, *e.g.*, the physiology of swallowing, the physiology of bladder emptying, intestinal motility, and cardiovascular studies, much of which is as yet unpublished.

The idea of cinefluorography seemed to be ideal for the study of eructation in small ruminants. The technique is relatively new and must still be considered to be in the experimental stage. There are only a few places where such studies can be made. The Radiology Department of the University of Rochester Medical School has done and is doing much of the original work in this field. Their splendid cooperation made possible the work herein reported. All of the cinefluorographic studies were made in the Radiology Department of the University of Rochester Medical School using sheep surgically prepared by the present authors.

From the Department of Physiology, New York State Veterinary College, Cornell University, Ithaca.

The authors wish to acknowledge the splendid cooperation of members of the Department of Radiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

This work was supported largely by a grant from the Animal Disease and Parasite Research Branch of the Agricultural Research Service, U.S.D.A.

PROCEDURE

Young western range sheep possessing permanent rumen fistulas were used in these experiments. Care was taken to select small and healthy animals and a somewhat restricted feeding regime was followed in order that they would be carrying as little excess condition as possible. A few days prior to the experiment, the sheep were closely shorn over the thoracic and abdominal areas.

The apparatus used is described more fully elsewhere.[7,8] It consists basically of a General Electric KX-1 generator and CRT 1-2 high energy output tube, making possible exposures at a maximum of 125 kilovolts, milliamperage values varying from approximately 40 to 80 and a time limit of 18 to 25 seconds when using a camera speed of 15 frames per second. Considerable experimentation was necessary to ascertain the optimum values but once determined it was possible to duplicate results fairly consistently provided cognizance was taken of variation in body weight from sheep to sheep.

The sheep were restrained in a special crate devoid of side members and designed to allow the animal to be brought into close contact with the fluoroscopic screen. This arrangement is seen in figure 1. Visual fluoroscopy was employed to facilitate positioning. A 14 by 14 inch DuPont-Patterson E2 screen (at target screen distance of 28 in.) was used, but due to the camera film gate being rectangular, an area of 12 by 14 inches was photographed with the long axis either vertical or horizontal as desired. Increasing screen-camera distance enabled the entire fluoroscopic area to be recorded but this naturally did not employ the entire film width and a smaller image was obtained. The electrically driven camera, synchronized with the primary generator circuit so that radiation was interrupted while the shutter was closed, employed 35 mm. kodak linagraph ortho film and was capable of speeds of 7.5, 15, 30, and 60 frames per second. Fifteen frames per second was found to be the best compromise between length of scene and adequate arrest of motion, the higher camera speeds giving proportionately shorter film exposure and therefore requiring energy output to be increased to give a correspondingly brighter fluoroscopic image. This in turn lessened the maximum length of the radiation period due to the heating of the tube.

It must be emphasized that in work of this nature the limitation of 25 seconds per scene enforced by tube heating is a severe disadvantage

and further exploitation of this medium will have to await solution of this problem.

Special lenses are essential and that used in this apparatus was a kodak fluro-ektar with f. aperture of 0.75. In the interests of better definition this was stopped down to f. 1.5.

The master 35-mm. negatives obtained were optically printed down to 16-mm. size and spliced into loops for continuous projection and study. It will be realized that the numerous stages involved in reproduction of the illustrations for this paper inevitably result in much loss of detail, excessive contrast, and grain.

A carbon dioxide (60 %) methane (40 %) gas mixture was insufflated into the rumen via the cannula fistula, the sheep being positioned with the right side against the fluoroscopic screen in order that the cannula did not contact its surface. Immediately prior to each run, the sheep were dosed with 10 to 20 ml. of thick barium sulphate paste to delineate the esophagus and cardia more clearly, early runs having shown that the gas entering the esophagus, and the esophageal wall itself, provided insufficient contrast for the detail desired.

Recordings were made of the normal ruminal cycle without any insufflation pressure and again using a pressure of 30 to 40 mm. of mercury to stimulate the eructation reflex. Rumen motility was recorded through the cannula-fistula on an ink-writing kymograph so that the scenes could be selected at chosen points in the rumen motility cycle.

RESULTS

Under these conditions eructation occurred immediately following the second reticular contraction of the ruminal cycle and commenced when the reticulum was fully relaxed. The rumino-reticular fold remained contracted and acted as a dam restraining the ingesta, while allowing the gas to come well down into the reticulum (fig. 2b), presumably under the influence of the caudally spreading ruminal contraction wave. This wave could not be observed due to limitation of the field. The cardia and another zone of constriction some 2 inches anterior to it then relaxed, or dilated, admitting the gas to the thoracic esophagus (fig. 2b and 2c). This was repeated rapidly, the cardia and "diaphragmatic" sphincter alternately closing and opening. The term "diaphragmatic sphincter" has been applied by several workers[9,10] to the extra sphincter or sphincters located in the thoracic esophagus (of man, dog, cat, and rabbit) at or near the diaphragm, not necessarily at the hiatus esophagus. For convenience, the anterior esophageal constriction observed in the sheep is termed "diaphragmatic sphincter" (fig. 2c) although it is in reality situated some distance cranial to the esophageal hiatus.

The barium sulphate introduced invariably fell into the rumen immediately caudal to the rumino-reticular fold and was usually carried forward into the reticulum with the ingesta that filled the reticulum after each major cycle of movement. From there it passed very rapidly into the omasum and appeared to become concentrated in this organ, often within forty-five minutes of administration. Some barium became dispersed in the ingesta of the rumen and increased its radio-opacity.

To test this mechanism of clearing the

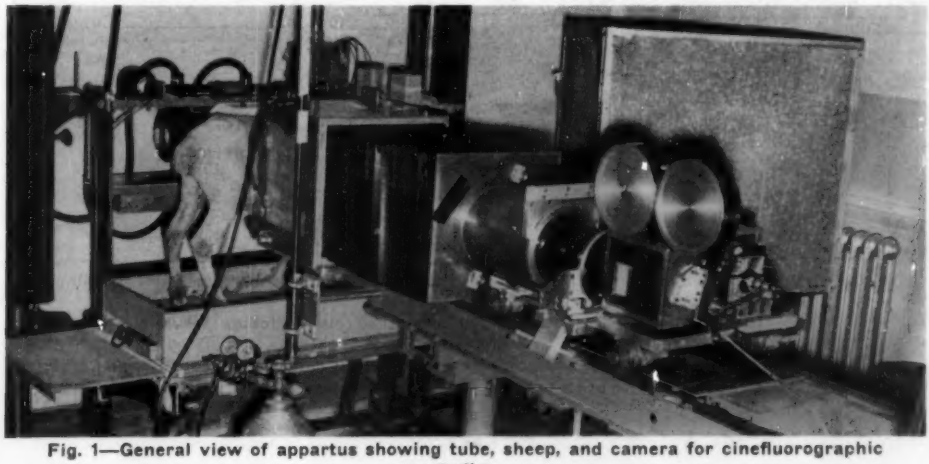

Fig. 1—General view of appartus showing tube, sheep, and camera for cinefluorographic studies.

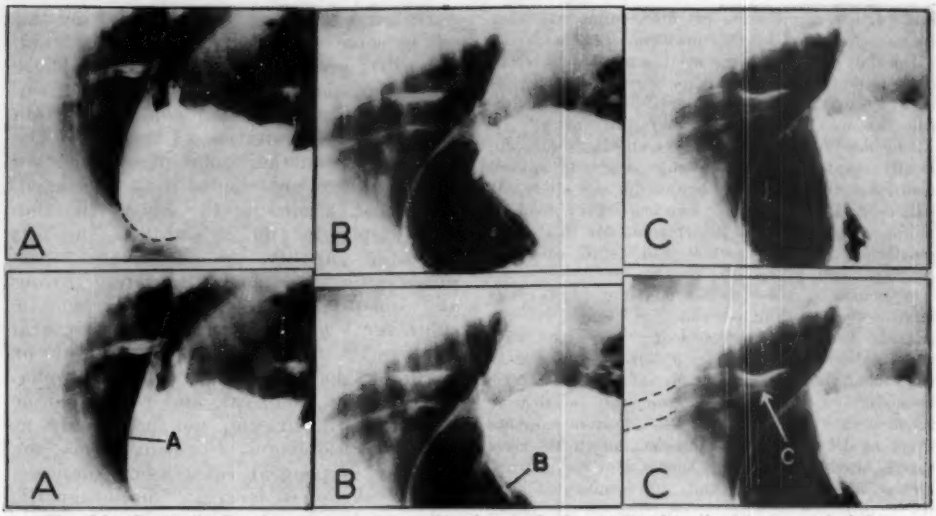

Fig. 2A—Partially filled rumen of sheep showing reticular wall with diaphragm at height of second contraction.

Fig. 2B—Relaxation of reticulum and dilation of esophagus and cardia showing ruminoreticular fold.

Fig. 2C—Relaxation of reticulum and dilation of esophagus and cardia showing the "diaphragmatic sphincter."

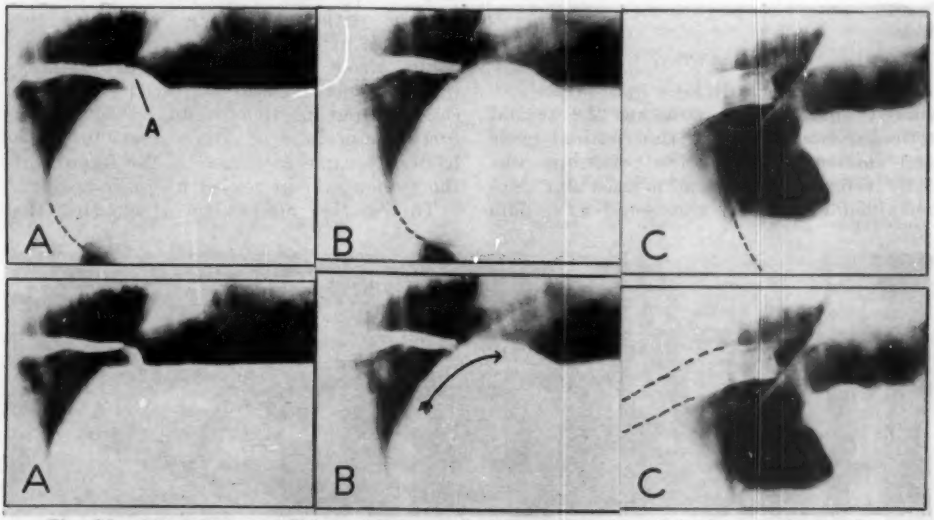

Fig. 3A—Attempted overfilling of rumen of sheep; A—bolus of barium paste passing through cardia.

Fig. 3B—Peak of second contraction of reticulum; arrow indicates wave of watery ingesta.

Fig. 3C—Relaxation of reticulum with ruminoreticular fold damming back fluid, some of which is seen spilling over into the reticulum.

cardia of ingesta and moving the gas pocket forward to the cardia, several sheep were filled as full as possible with water and the rear of the restraining crate elevated to cover the cardia with fluid. Gas pressure as before was applied and it was found that even when the cardia was on a level with or just covered by fluid, the ability to lower the anterior (*i.e.*, reticular) fluid level and thus clear the cardia for eructation was retained. It was difficult to cover the cardia completely as the weight of water introduced stretched the abdominal musculature and lowered the fluid level, and the gas pressure applied above the ingesta mass accentuated this effect (fig. 3a). This was especially marked when the rumen was relatively empty as the depression of the ingesta level occasionally even permitted the entry of gas into the abomasum.

Use of an acetylcholine blocking agent * caused complete cessation of rumen motility but did not affect that of the esophagus and cardia. Eructation occurred even when overfilled with water but, as had been observed before, it was difficult to occlude the cardia under these conditions and presumably had the fluid level been higher, eructation would have been impossible due to entry of the rumen contents into the thoracic esophagus. When small amounts of fluid did enter the thoracic esophagus (fig. 4), they were swallowed before the next eructation. Only the thoracic part of the esophagus was visible in the viewing area, but it was assumed that liquid ingesta did not reach the pharynx or oral cavity.

Administration of epinephrine hydrochloride intravenously completely inhibited the rumen and reticulum. The cardia and esophagus (except for deglutition) remained inactive for the 20-second period of observation, and no movements of eructation occurred even under the influence of rumen insufflation.

To determine whether raised intra-abdominal pressure or anterior displacement of the diaphragm affected the circulation, an angiocardiogram was taken on one sheep. The right jugular vein was isolated under local anesthesia and a large bore polyethylene catheter was passed down to the right atrium. To obtain a control recording, approximately 30 ml. of urokon † contrast

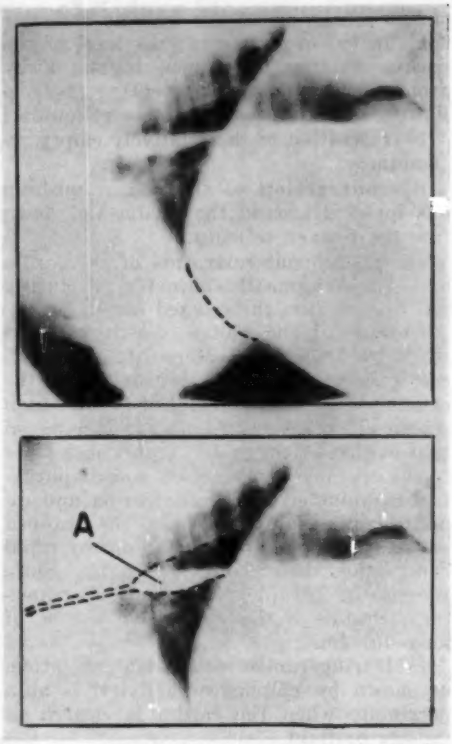

Fig. 4—Entrance of fluid into thoracic esophagus and its return to rumen by peristalsis; A—barium and water mixture.

medium was injected which gave a perfect outline of heart, pulmonary circulation, and aorta. A pressure of 40 mm. of mercury was applied intraruminally and a subsequent injection of 40 ml. of thorotrast ‡ made. Unfortunately, animal movement and forward displacement of the heart put the viewing area partly off the screen but, apart from some tachycardia, no gross alteration of circulation time or distortion of the great vessels was discernible.

Discussion

A number of mechanisms were shown, in this work, to be especially important in eructation:

a) the clearing of ingesta from the reticulum by two contractions of this organ;

* Compound oc. 1052-1575, Sharp & Dohme, Inc.

† Uroken sodium, Mallinckrodt Chemical Works, St. Louis 7, Mo.

‡ Heyden Chemical Corporation, 393 Seventh Ave., New York 1, N. Y.

b) contraction of the rumino-reticular fold up to (or nearly to) the level of the cardia, holding the rumen ingesta away from the cardia and preventing it from flowing or falling back into the reticulum;

c) relaxation of the relatively empty reticulum;

d) contraction of the rumen pushing gas forward around the cardia and down into the relaxed reticulum;

e) synchronous relaxation of the cardia and diaphragmatic sphincter permitting gas to rush into the relaxed esophagus.

Closure of the glottis shown in other work by Dougherty, Meredith, and Barrett [11] occurred at the beginning of the eructation reflex. Although it has not been shown cinefluorographically, it is assumed that closure of the glottis occurs just prior to the opening of the cardia and diaphragmatic sphincter. After the cardia and diaphragmatic sphincter close, the trapped gas in the esophagus is forced out by rapid contraction throughout its length. Since the rise in intrapleural pressure is transitory, closure of the glottis can not be of long duration.

f) During rumen insufflation eructation, as shown by esophageal activity, is at a maximum when the cardia is cleared of ingesta or fluid.

It is felt that this and other work [11] have established a working knowledge of the mechanism of eructation in sheep. The same mechanism may exist in other ruminants. The important missing information in the knowledge of eructation is the type and location of receptors that initiate the eructation reflex and complete knowledge of the innervation of the esophagus and cardia. Evidence showing the presence and the location of an eructation center will be of academic interest assuming, of course, that such exists.

There is still much to be done on motility problems, but cinefluorography is expensive and until longer recording periods are feasible it is doubtful if sufficient data can be obtained to justify the outlay. Recent developments indicate that longer exposure time and greater definition can be expected within the next few years.

It would appear that motility of the rumen and reticulum is not essential to eructation provided the cardia is not covered by ingesta, and it is unlikely, in the absence of foaming of the ingesta or severe overfilling, to be covered especially when there is distension with gas. What effect foaming has on the reticular mechanism described remains to be seen, but presumably it would make eructation more difficult, particularly if foam covered the cardia. Conversely, in the event of overfilling and/or frothing of the ingesta the absence of the reticular mechanism for clearing the cardia could conceivably prevent eructation completely even if the cardia itself is not inhibited.

SUMMARY

1) Cinefluorographic studies were made of normal activity of the esophagus, reticulum, and rumen of sheep.

2) The same type of study was made of the same animals under conditions of rumen insufflation.

3) Detailed studies were made of the structures involved in eructation.

4) Through the use of contrast mediums, an attempt was made to study the effect of rumen insufflation on the heart and circulation in the thoracic region.

5) The sequence of events occurring during eructation in sheep is presented.

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Further Research on the Prevention of Kidney Stones in Sheep from the Colloidal Approach

G. O. PUNTRIANO, D.V.M., M.S.

Laramie, Wyoming

A PREVIOUS article,[1] reported the important role played by the protective urinary colloids in the etiology of urolithiasis in livestock and that nutrition may constitute one of the chief factors in determining the concentration of protective colloids in their urine. To investigate the latter, several groups of sheep were fed a urinary calculi-predisposing ration, with or without hyaluronidase pellets, and its effects on the concentration of protective urinary colloids and on the incidence of calculi were determined.

MATERIALS AND METHODS

Based on an experiment reported by Tayson *et al.*[2] which indicates that when 25 to 40 per cent beet pulp was included in the ration it was considered responsible for the incidence of urinary calculi in a sheep, we prepared a urinary calculi-predisposing ration composed of 50 per cent beet pulp, 25 per cent rolled barley and 25 per cent wheat bran. Alfalfa hay was fed free choice.

The feeding experiment was conducted for five months using 24 lambs—20 wethers and 4 rams averaging 7 to 9 months of age. All were vaccinated, subcutaneously, with 5 cc. of *Clostridium perfringens* type D vaccine at the beginning of the experiment, were tagged on the left ear, and then separated into four groups with groups A, B, and C consisting of 6 wethers each, and group D of 2 wethers and 4 rams.

Each lamb of groups A, B, and C received 1 lb. of the urinary calculi-predisposing ration daily for a month, after which each was allowed the same ration free choice. Each lamb of group A also received a pellet containing 3,000 reducing turbidity units of hyaluronidase as a subcutaneous implant in the neck, at the beginning of the second, third, and fourth month of the experiment. The hyaluronidase pellets were manufactured by the author using a commercial hyaluronidase and a waxy substance as a base.

Group D served as a control and received only alfalfa hay, free choice. Fresh water was available at all times.

Urine samples from each lamb were collected once a week during the first month of the experiment and thereafter, every four days by means of a rubber bag vulcanized to a rubber band, made from an inner tube, which was tied to the abdomen of the lamb (fig. 1 and 2).

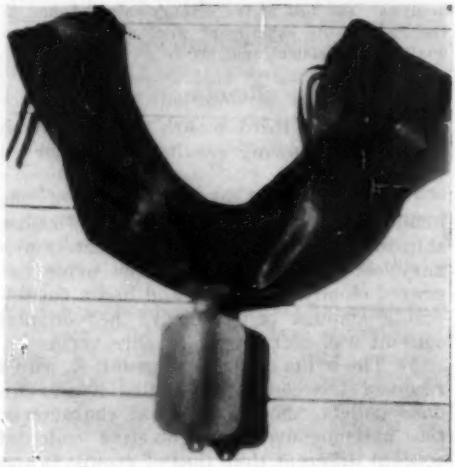

Fig. 1—The rubber tube, with bag attached, for collecting urine.

Each urine sample was checked for physical characteristics, pH, predominant crystals in dried films of urine, and for the average protective colloidal content using the Ultropak microscope. The technique and details of the two latter examinations have been discussed in a previous article.[2]

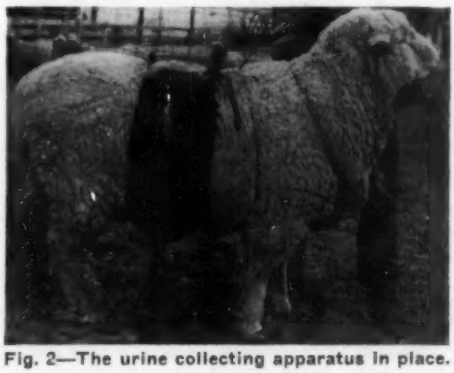

Fig. 2—The urine collecting apparatus in place.

From the Wyoming State Veterinary Laboratory, Laramie.

Part of this paper was presented at the Twenty-Sixth Annual Meeting of the Intermountain Veterinary Medical Association at Salt Lake City, Utah, Jan. 18, 1954.

All the sheep were killed at the end of the experiment (150th day) and their kidneys and urinary tracts were carefully examined and checked for the presence of calculous material. Some of the "stones" found during this examination, as well as a calculus recovered from wether 214 of group C on the ninety-eighth day of the experiment, were submitted to a quantitative chemical analysis. The rest of the urinary calculi found at the end of the experiment were submitted to a qualitative chemical analysis.

RESULTS

During the third month of the experiment, the following results were observed (table 1):

1) The physical characteristics of the urine in every group showed some relationship to the concentration of protective urinary colloids. Thus, when the urine appeared cloudy or opaque and had a considerable amount of sediment, the colloidal content was decreased; and vice versa.

2) The urine of sheep in group A, which received the concentrates plus hyaluronidase pellets, showed physical characteristics and an average protective colloidal content different than that of groups B and C, group A having a well preserved crystalloid-colloid balance.

3) The urine of sheep in groups B and C, which received the same ration but no hyaluronidase, showed very similar physical characteristics and average protective colloidal content, the presence of crystalline concretions and decreased protective urinary colloidal content suggesting crystalloid-colloid imbalance in their urine.

Table 2 shows the percentage content of several minerals of the ration supplied to the sheep according to Morrison;[3] the Ca/P ratio of the ration being normal.[4]

TABLE 2—Percentage Content of Some Minerals of the Ration Supplied to the Sheep*

	Calcium (%)	Phosphorus (%)	Magnesium (%)	Silicon (%)	Proportion in the ration (%)
Beet pulp	0.68	0.07	0.26	0.13	50.0
Wheat bran	0.12	1.32	0.55	0.03	25.0
Rolled barley	0.05	0.38	0.12	0.15	25.0
Alfalfa hay	1.43	0.21	0.26	0.25	Free choice

* According to Morrison[3]; Ca/P ration: 2.3/1 approximately.

Table 3 shows the results of the qualitative chemical analysis of the sediment from urine of sheep during the third month of the feeding experiment. Silicon was the only element constantly found in all the determinations.

Table 4 shows the results of the qualitative chemical analysis of concretions and sand-like calculous material found in the kidneys of wethers 207 and 209 of group

TABLE 1—Results of Tests on Urine from Sheep During the Third Month of the Experiment

Group	Treatment	Physical Characteristics: transparency	Physical Characteristics: sediment	Range of pH	Predominant crystals in dried films-urine	Average colloidal content of protective colloids
A	Ration of concentrates and alfalfa hay, plus hyaluronidase pellets subc. at the begining of 2nd and 3rd month	Mostly clear; some were cloudy	Little or none	7.2 to 8.6	Calcium carbonate and triple phosphate	Sufficient concentration providing very good protection to the urinary crystalloids
B	Ration of concentrates and alfalfa hay	Mostly cloudy or opaque	Considerable	7.3 to 8.9	Calcium carbonate and triple phosphates; few crystalline concretions of unidentified material	Insufficient concentration; evidence of crystals deprived of colloidal protection
C	Ration of concentrates and alfalfa hay	Mostly cloudy and opaque	Considerable	7.0 to 8.2	Calcium carbonate and triple phosphates; many crystalline concretions of unidentified material with a tendency to aggregate	Insufficient concentration; evidence of many crystals deprived of colloidal protection, especially the crystalline concretions
D	Ration of alfalfa hay only (control)	Mostly clear	Little or none	7.6 to 8.6	Triple phosphates	Sufficient concentration providing satisfactory colloidal protection to the urinary crystals

TABLE 3—Qualitative Chemical Analysis of the Sediment from Urine of Sheep During the Third Month of the Experiment

Sheep (No.)	Ca	PO_4	Mg	Si	CO_3	C_2O_4
			GROUP B			
207	+	−	−	+	+	−
208	−	−	+	+	−	+
209	−	+	−	+	−	−
210	+	−	−	+	+	−
211	−	+	−	+	+	+
212	+	−	+	+	−	−
			GROUP C			
213	+	−	−	+	−	−
214	−	+	−	+	−	−
215	+	−	+	+	+	+
216	−	+	−	+	−	−
217	−	−	−	+	+	+
218	−	+	+	+	−	−

+ Component present; − Component absent.

B, and wethers 213 and 216 of group C, when they were killed at the end of the experiment. Silicon and protein were the elements constantly found in all the determinations.

Table 5 shows the results of the quantitative chemical analysis of the urinary calculi recovered from wethers 214 and 218 of group C, and from wether 211 of group B. When wether 214 showed clinical symptoms of urolithiasis on the ninety-eighth day of the experiment, he was killed and occluding urethral calculi were recovered. The calculi from the other 2 wethers appeared like sand or concretions and were recovered from the kidneys at the end of the experiment. The high percentage of silicon in each indicates that the urinary calculi were of the silicon type.

The sheep in groups A and D, plus those not mentioned above in groups B and C, did not show evidence of calculous material in their kidneys or urinary tracts at the end of the experiment.

It thus appears that the sheep in group A, which received hyaluronidase and concentrates, maintained the crystalloid-colloid balance in their urine without the incidence of urinary calculi while the sheep in groups B and C, which received the same ration but no hyaluronidase, showed a crystalloid-colloid imbalance in their urine, and some urinary calculi. The sheep in group D (control) which received neither concentrates nor hyaluronidase maintained the crystalloid-colloid balance in their urine and did not show urinary calculi.

TABLE 4—Qualitative Chemical Analysis of the Urinary Calculi of Sheep

Sheep (No.)	Ca	PO_4	Mg	Si	CO_3	C_2O_4	Protein
			GROUP B				
207	+	+	−	+	+	−	+
209	−	+	−	+	−	+	+
			GROUP C				
213	+	−	+	+	−	−	+
216	+	+	−	+	+	−	+

+ Component present; − Component absent.

DISCUSSION

The results of this feeding experiment suggest that the mineral content of the concentrates given to the sheep in groups A, B, and C was responsible for the crystalloid-colloid imbalance in the urine, as well as for the urinary calculi but that the hyaluronidase treatment given to group A helped to maintain the crystalloid-colloid balance in the urine and prevented the incidence of urinary calculi, in that group, by supplying an extra amount of protective colloids.

TABLE 5—Partial Quantitative Chemical Analysis of the Urinary Calculi of Sheep

Sheep (No.)	CaO (%)	P_2O_5 (%)	Mg (%)	SiO_2 (%)	Ash (%)
		GROUP B			
211	.....	0.45	0.12	62.1	75.3
		GROUP C			
214*	0.48	0.82	.....	75.4	81.5
218	0.53	0.79	0.15	71.2	74.0

* Calculi recovered from this wether on the ninety-eighth day of the experiment.

The fact that the Ca/P ratio of the ration supplied to the sheep in groups B and C was normal [4] suggests investigation of the possible role played by magnesium and silicon in the incidence of urolithiasis and in the crystalloid-colloid imbalance of the urine of sheep in these groups.

While magnesium was infrequently found in the urinary sediment and calculous material of several wethers in groups B and C, silicon was constantly found in all the determinations. Furthermore, the fact that the calculi recovered from several wethers in groups B and C were of the silicon type, indicates that silicon was the mineral mainly responsible for causing the crystalloid-colloid imbalance in their urine and also for causing urinary calculi in some of the wethers of these groups.

The mechanism whereby silicon forms calculi in the kidneys or urinary tracts of sheep has not been determined. Swingle [5] suggested that in livestock the "stones"

are probably the result of a precipitation of the silica by the mucoprotein of the urine. He adds that while the conditions determining whether the precipitate is dispersed or is gathered into concretions are not known, he suspects at least two factors: (*a*) the presence of particulate nuclei and (*b*) the alterations in the quality and quantity of protective colloids in the urine.

In the author's opinion, an excess of silicates in the urine of sheep would upset the crystalloid-colloid balance either by: (1) remaining as silicates which, when in excess, would increase the proportion of urinary crystalloids, or (2) changing into silic acid (nonprotective colloid) and, thereby, abnormally increasing the proportion of nonprotective colloids in the urine.

The chemical reaction, of urinary silicates with the buffering substances of the urine, would result in the formation of silic acid. This reaction would be more likely when the buffering substances are in increased proportions in the urine, as occurs when a diet rich in proteins, such as was used in this experiment, exerts its effects on the acid-base balance of the body.[6]

The formation of silic acid in the urinary tract of sheep has special significance because this nonprotective colloid readily forms a gel which would serve as a nucleus or as a cementing material to which any urine crystalloids, which were deprived of protective colloids, would aggregate and grow to form calculi of clinical significance.

In our experiment, the silicon type of calculi found in the sheep probably had its origin in a nuclei of silic acid. Both the excess of silicon eliminated in the urine and the buffering substances of the urine, by chemically reacting, probably resulted in silic acid formation and then in calculi.

SUMMARY

The effects of a urinary calculi-predisposing ration were tested on three groups (A, B, and C) of 6 lambs each. The 6 lambs of control group D received alfalfa hay only. Fresh water, free choice, was supplied to all the sheep.

The sheep in group A which received a treatment with hyaluronidase pellets, implanted subcutaneously in the neck, did not show crystalloid-colloid imbalance in their urine, nor incidence of urinary calculi while those in groups B and C showed crystalloid-colloid imbalance in their urine and some developed urinary calculi. The sheep in group D (control) showed neither crystalloid-colloid imbalance nor urinary calculi.

It is proposed that the excess of silicon eliminated in the urine was responsible for the crystalloid-colloid imbalance and urinary calculi which developed in the urine of sheep in groups B and C and that the hyaluronidase treatment given to group A helped to maintain the crystalloid-colloid balance in the urine and to prevent the incidence of urinary calculi by supplying an extra aomunt of protective colloids.

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Observations on the Life Cycle of *Nematodirus Spathiger*, a Nematode Parasitic in the Intestine of Sheep and Other Ruminants

K. C. KATES, Ph.D., and J. H. TURNER, M.S.

Beltsville, Maryland

SEVERAL species of trichostrongylid nematodes of the genus Nematodirus have long been known as parasites of domestic ruminants.

They had often been suspected of causing parasitic disease prior to the experimental demonstration of the pathogenic effects of *Nematodirus helvetianus* in calves by Herlich and Porter,[6] and of those produced by *Nematodirus spathiger* in lambs as shown by the writers.[7] The literature on the pathogenicity of various species of Nematodirus in ruminants has been summarized by the afore-mentioned authors, except for the recent reports of Crofton and Thomas[2] and Kingsbury,[5] who presented strong evidence that another species of the genus, *Nematodirus battus*, probably was responsible for heavy losses of lambs in Great Britain.

Prior to 1954, only one life cycle study of a Nematodirus species had been reported, namely, that of *Nematodirus filicollis* in sheep by Boulenger,[1] who studied the free-living stages and some parasitic stages obtained from naturally infected sheep. Recently, however, Herlich[5] described in more detail the life cycle of *N. helvetianus* of cattle, and summarized certain fragmentary observations recorded in the literature pertaining to some aspects of the life cycle of various species of the genus. One of us (Turner[21]) investigated the survival of eggs and larvae of *N. spathiger* after exposure to various pasture and laboratory conditions and presented further evidence that the free-living stages of this species are unusually resistant to freezing, desiccation, and variable climatic conditions.

The objectives of this paper are to present a brief description of the life cycle of *N. spathiger* to show the relationship of the various parasitic stages of the nematode in lambs to the clinical symptoms and intestinal pathology resulting from massive experimental infections and to compare our observations on *N. spathiger* with those of other workers on other species of the genus. This report supplements a previous one on the same parasite by the writers.[7]

MATERIALS AND METHODS

In order to study the development of larvae within the egg membranes, eggs were obtained from sheep feces by flotation on concentrated sugar solution. These eggs were then washed in water and cultured in distilled water in covered watch glasses in the laboratory at an average temperature of about 75 F. At frequent intervals until development was complete, some of the eggs were removed with a pipette, placed on a slide, and examined. From similar preparations, photomicrographs were made of the developing larvae at various intervals after the eggs had been cultured.

Parasitic larvae were obtained for study from lambs, 2 to 6 months old, after each animal had been given a single dose of either 825,000 or 1,000,000 infective larvae. Infective larvae were obtained from cultures and given to the lambs as previously described by the writers.[7] The large doses of larvae were used in order to be certain that a sufficient number of larval parasites would be found in histological sections of the intestines in order that their normal position in the mucosa or lumen could be determined. Also, by this procedure, a large enough infection could be established to elicit the maximum pathological changes in the intestines by the parasites.

The 6 infected lambs were kept in large animal cages and fed adequate quantities of alfalfa hay and mixed grain during their relatively short stay therein. The lambs were weighed and blood samples obtained for routine hematological studies on the day the larvae were administered, at regular intervals thereafter, and just before the animals were killed. The lambs were killed for study as follows: 2 on the fifth day after having been given larvae, 1 on the eighth day, 1 on the tenth day, 1 on the twelfth day, and 1 on the fifteenth day. Thereafter, a uniform procedure was followed to obtain pertinent information on the infections and on tissue and parasite specimens for study. Within five minutes or less after the lambs were killed, the abdominal cavity was opened and the small intestine tied off with string near the pyloric and ileocecal valves. After this was done, 3 1-in.

From the Animal Disease and Parasite Research Branch, Agricultural Research Service, U.S.D.A., Beltsville, Md.

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sections of the small intestine, located 10, 12, and 14 ft. (intestinal distance), respectively, from the pyloric valve, were tied off, severed from the rest of the intestine, and dropped into vials containing Bouin's fluid for immediate fixation; serial, transverse sections 8 to 10 μ thick were later made from these pieces of intestine, and stained with hematoxylin and eosin.

After the 1-in. sections were obtained, the small intestine was removed from the animal and a piece of the jejunum several inches long, likely to contain a high concentration of worms, was excised, placed in warm physiological saline solution in a large Petri dish, incised lengthwise, and the appearance of the mucous membrane and position and activity of the worms studied with the aid of a dissecting microscope. In this manner, the gross changes in the mucous membrane and the living larval and adult parasites *in situ* were studied as soon as possible after the lambs were killed. After this had been done, all the worms in the intestine were recovered, except those present in the three small pieces fixed for later microscopic study, and stored in 70 per cent alcohol for later study and counting. Total counts of worms were made by a routine dilution method. Most preparations for microscopic study of the parasitic larvae were temporary mounts made from uncleared, alcohol-fixed specimens. Certain details not clearly seen in alcohol-fixed specimens were also studied in living specimens mounted in physiological saline solution. All drawings were made with the aid of a camera lucida.

RESULTS

Preparasitic Development.—The morphology, measurements, and other features of the eggs and infective larvae of *N. spathiger* have been described in the literature.[5, 10, 11] A brief description is included herein of the preparasitic development. The pattern of development from egg to infective larva of *N. spathiger* is essentially similar to that of *N. helvetianus* as described by Herlich.[5] Whereas Herlich [5] illustrated his description of the preparasitic stages of *N. helvetianus* with drawings, the writers obtained photomicrographs of the preparasitic stages of *N. spathiger* (plate 1).

The eggs of *N. spathiger* are slightly smaller on the average than those of *N. helvetianus*, the mean length of the former being about 200 μ and mean width about 98 μ; the eggs of these two species are larger than those of *N. filicollis*.[10] Generally, the eggs of the afore-mentioned three species, and others of the genus, closely resemble one another. When *N. spathiger* eggs are passed in the feces, they contain two to eight large, coarsely granular cells (fig. 1). The development of the larvae to the infective or third-stage takes place within the egg membranes. The larvae do not emerge from the egg membranes or "hatch" until they are infective to sheep. The sheaths of the first and second molts are retained until hatching occurs, whereupon the cuticle of the first molt is shed, the larvae normally retaining the cuticle of the second molt until they are ingested by the host, as noted by Herlich [5] for *N. helvetianus*. Because of this a precise determination of the time of completion of the first molt is difficult. However, in our cultures the cuticle of the first molt was first seen after eggs had been cultured for five to six days (fig. 4), the larvae at this time being in the early second stage. Prior to the first molt the larvae passed through typical morula and tadpole stages (fig. 2 and 3). During the period of six to fourteen days in culture, second-stage larvae transformed into infective or third-stage larvae and the sheath of the second molt was formed (fig. 5). However, infective larvae commonly did not begin to hatch until about fourteen days in culture (fig. 6 and 7), and normally hatching did not progress at a uniform pace, continuing for a week or more in the same batch of eggs, because some developed more slowly than others and some were delayed in hatching after reaching the infective stage. Once the egg membranes had been ruptured, the emergence of the infective larvae took place rapidly, only a few seconds elapsing between the first appearance of a break in the egg membranes and the complete emergence of the larvae (fig. 6 and 7). Some or all of the delicate cuticle of the first molt shed at this time was left behind in the empty shell. The preparasitic development of *N. spathiger* is similar in these respects to that of *N. helvetianus*, as reported by Herlich.[5] It should be noted, however, that when Herlich maintained egg cultures at about 85 F., hatching of the larvae began between the eighth and tenth days of culture, and practically all larvae had hatched by the end of the third week.

Parasitic Development.—Specimens for study of the parasitic stages were obtained from 6 lambs killed at various intervals after having been given single large doses of infective larvae (table 1). Although all these lambs were maintained under similar

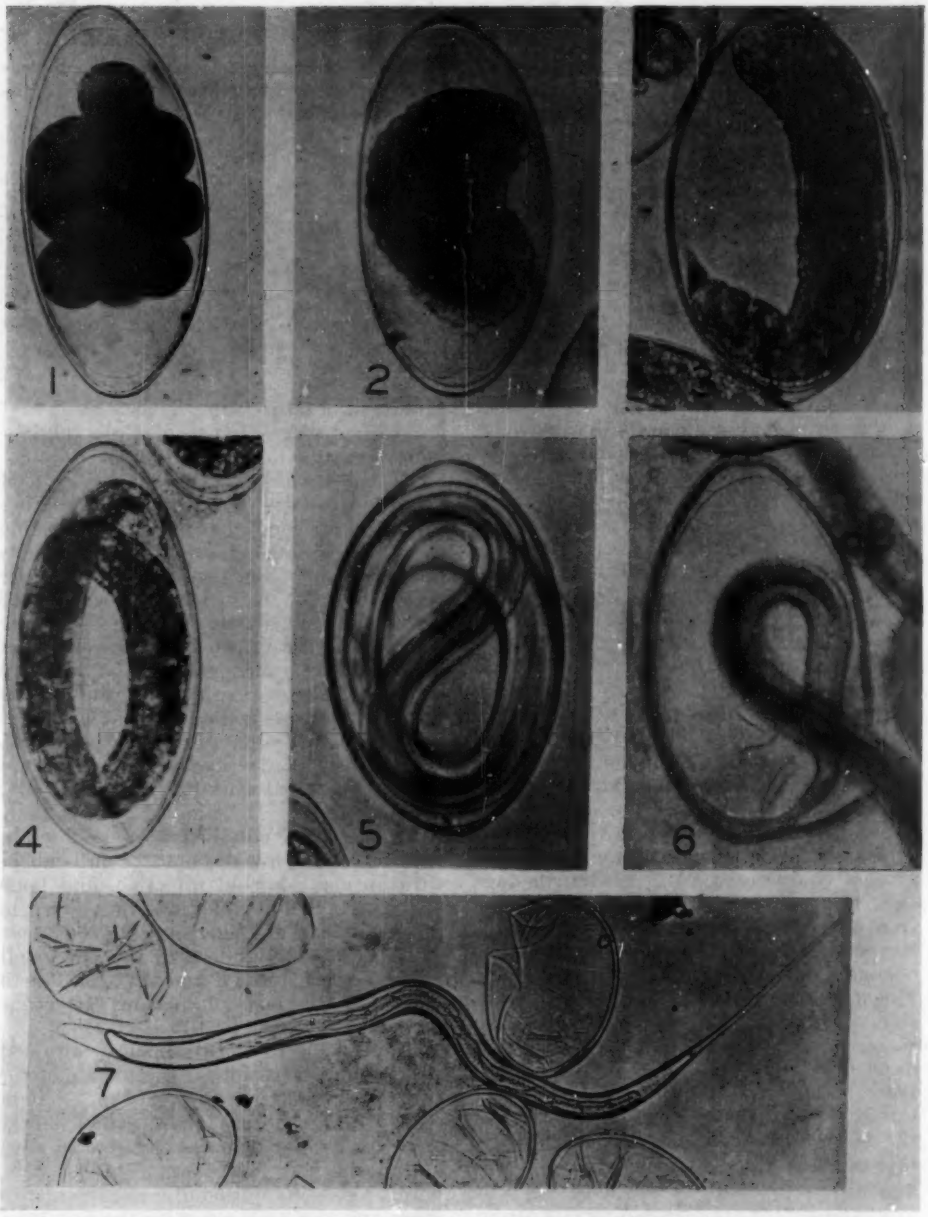

Plate 1—Preparasitic development of Nematodirus spathiger; photomicrographs of living specimens. Magnification: Fig. 1 to 6 approximately 280; fig. 7 approximately 140.

Fig. 1—Egg as voided by female or in fresh feces.

Fig. 2—Late segmentation, two-day culture.

Fig. 3—Early first-stage larva, four-day culture.

Fig. 4—Second-stage larva, six-day culture.

Fig. 5—Transitional between second and third stage, ten-day culture.

Fig. 6 and 7—Infective, third-stage larva, fourteen-day culture.

Fig. 6—An infective larva emerging from "shell."

Fig. 7—Newly hatched infective larva and several empty "shells."

conditions and the larval doses administered in the same manner, 4 lambs retained much larger numbers of parasites at necropsy than did the remaining 2 lambs. However, ample specimens were obtained from all the lambs for the study of the parasitic development. The best material for study of the tissue changes in the small intestines and position of the parasites in the mucosa was obtained from the 4 lambs with massive infections at the time they were killed.

Third Molt, Fourth-Stage Larvae.—Since the majority of the immature worms recovered from five-day-old infections were in the early fourth stage of development, the third molt and the transformation of the majority of third-stage larvae into fourth-stage larvae must have occurred prior to the fifth day of parasitic life, probably between the third and fifth days. However, a few early fourth-stage larvae, which still retained the cuticle of the third molt, were recovered from the five-day infections (fig. 14). These larvae averaged 1.3 mm. long and 0.05 mm. wide at their greatest width. The external appearance of larvae in the third molt was similar to that of the infective larvae minus the cuticle of the second molt, in that the tail of the retained cuticle of the third molt was two-lobed with a median process. Larvae within the cuticle of the third molt had typical blunt tails with a single terminal process characteristic of the fourth-stage (fig. 14 B). During the period of growth from infective larvae to early fourth-stage larvae in the third molt, considerable change occurred in the internal structures. The digestive system had undergone considerable growth, the esophagus having become more robust and longer, and the number of intestinal cells had increased from eight to many times this number. Ganglion cells surrounding the midpart of the esophagus and undifferentiated cells in the tail had increased in number. The genital primordium was now about 0.1 mm. in length and contained many cells. However, the outstanding feature of larvae in the third molt compared with free-living, third-stage larvae in the second molt was the great increase in size of the excretory system, particularly the renette cells (excretory or cervical glands), which were two in number, each with a large nucleus (fig. 14 A). The renette cells are not only

TABLE 1—Recovery of Immature Nematodirus Spathiger at Necropsy from Lambs Administered Single Large Doses of Larvae

Lamb (No.)	Days after infection	Worms recovered**
158	5	8,000
199*	5	650,000
77	8	540,000
179	10	31,000
187*	12	500,000
79	15	396,000†

* Administered 825,000 larvae; other lambs 1,000,000 larvae.

** Worms in 3 1-in. sections of small intestine removed for sectioning not included.

† Less than 5 per cent mature.

prominent features throughout the parasitic larval stages but also in the adults,[9] and in larvae in the third molt they extended posteriorly almost to the genital primordium, one renette cell extending farther posteriorly than the other. In this stage and in later stages, the renette cells appeared to connect ventrally with the single excretory tube near the base of the esophagus. When stained sections of the jejunum of the infected lambs were studied, one of the obvious features of many sections of the parasitic larvae and adults was the pink, eosinophilic granules which filled the renette cells; these can be seen as finely granular, dark areas in some of the sections of nematodes in figures 10, 11, and 12.

All the larvae obtained from the 2 lambs with infections five days old, from lamb 77 with an eight-day-old infection, and lamb 179 with a ten-day-old infection, were in the fourth larval stage, showing that most of the larvae had undergone the third molt prior to the fifth day, and that the fourth molt did not take place until after the tenth day of parasitic life.

Figure 15 illustrates the youngest fourth-stage larvae, after completion of the third molt, recovered from the 2 lambs with five-day-old infections. These larvae varied in length from 2.4 mm. to 2.6 mm., and in width from 0.035 mm. to 0.05 mm., both sexes being of about equal size at this stage and distinguishable for the first time. The posterior end of the male was slightly thicker and contained more cells than the female. Both sexes had blunt tails with a single, pointed terminal process (fig. 15 A and B). The genital primordium had grown and in the female appeared as an elongate, sausage-shaped structure about 0.2 mm. long (fig. 15 A), whereas in the

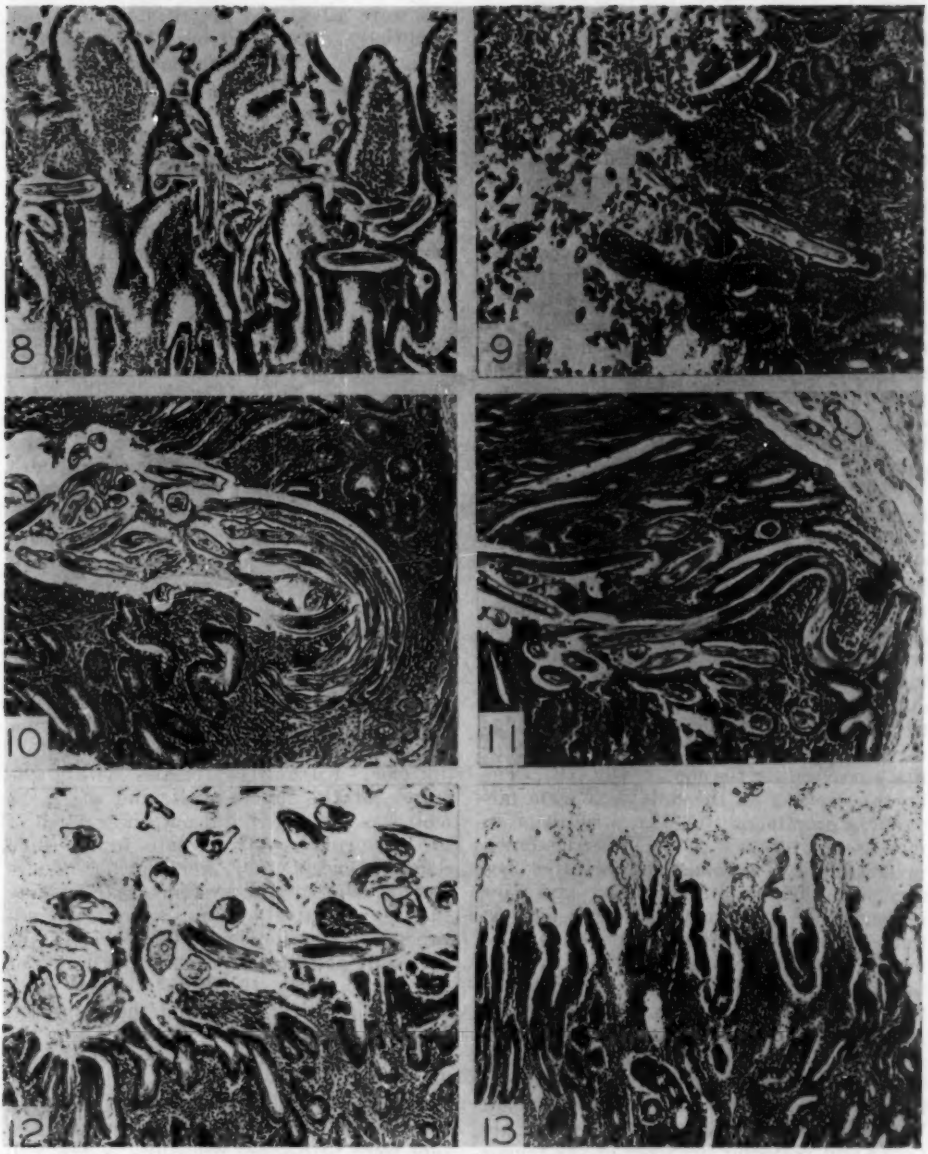

Plate 2—Photomicrographs of transverse sections of jejunum of infected lambs. Magnification all figures approximately 120.

Fig. 8 and 9—Five-day infection, lamb 199. Fig. 10 and 11—Eight-day infection, lamb 77. Fig. 12 and 13—Fifteen-day infection, lamb 79.

The surface of the mucosa in figures 8, 12, and 13 is at the top of each photomicrograph, in figures 9, 10, and 11 to the left.

male it had a more elongate, dumbbell shape, the overall length being about twice that of the female primordium. At the cephalic end a cuticular inflation, more or less typical of the adults of the genus, was seen for the first time. The intestinal cells were more numerous than in larvae during the third molt, and the longest of the two renette cells still extended posteriorly almost to the anterior end of the genital primordia. A few larvae from the five-day infection were somewhat more advanced than those illustrated in figure 15. Figure 16 shows the greatest development of the female reproductive system observed five days after infection; some of the cells of the genital primordium now formed the primitive vagina and others were beginning to form the anterior and posterior branches of the female reproductive system.

Fourth-stage larvae recovered from the lamb killed eight days after infection (fig. 17-20) were larger than those 5 days old, the outstanding feature being an increase in length, without a comparable increase in width. The females were 4 to 5 mm. long and 0.055 to 0.06 mm. in maximum width, whereas the males were only 3.1 to 4.2 mm. long and of similar width. The cephalic cuticular inflation and transverse striations thereof were more marked than before, and there were six small papillae or lips surrounding the mouth (fig. 19). The bursal swelling in the 8-day-old male larvae was prominent (fig. 17), a result of an increase in number of cells in the bursa primordium. The male reproductive system was further differentiated and now measured 0.8 to 1.0 mm. in length and extended posteriorly almost to the anus. The male reproductive system showed an anterior, club-shaped region, the precursor of the testis. The female reproductive system measured 0.7 to 0.8 mm. in total length (fig. 18), and the vagina, though more advanced in development than in the younger larvae, was not yet open to the exterior. The terminal ovarian regions of the anterior and posterior branches of the female system at this stage also were club-shaped. A lumen had not yet formed in any part of the reproductive systems of the two sexes in 8-day-old larvae, except in the vagina. The posterior ends of both sexes retained their blunt shape and single caudal process (fig. 17 and 20).

Ten-day-old larvae recovered from lamb 179 were all in the fourth stage and not essentially different from those obtained from the eight-day infection, except that some were slightly longer and slightly more advanced in development. The maximum length of the females was 7 mm. and the males 5 mm., but little change in width was noted. No larvae in the fourth molt were recovered from this lamb.

Fourth Molt, Fifth Stage (Preadults).—The majority of immature parasites recovered from lamb 187, with a twelve-day-old infection, were in the early fifth stage, having completed the fourth molt, but some were still undergoing the fourth molt (fig. 21 and 22) and some were still in the late fourth stage. The maximum length of the young fifth-stage females was 14.0 to 15.0 mm. and the maximum length of the males was 10.0 to 11.0 mm.; the maximum width of both sexes was 0.1 mm. By the twelfth day of parasitic life, therefore, some fifth-stage parasites were near the normal minimum length of adults, although no sexually mature specimens were recovered from lamb 187. The fact that fourth-stage larvae, larvae in the final molt, and young fifth-stage worms were recovered from the twelve-day-old infections showed that the pace of development of the nematode population established by administering a single large dose of larvae was not entirely uniform. Fifth-stage females within the cuticle of the fourth molt, and after the molt was completed, did not differ much in gross morphology from late fourth-stage females, except they were larger in size and various organ systems, particularly the reproductive system, were more advanced in development. However, in the male just prior to and during the period of the fourth molt, the bursa, bursal rays, and spicules developed, and the blunt tail with its caudal process was lost when the cuticle of the fourth molt was shed (fig. 22).

The majority of the worms recovered from lamb 79 with a fifteen-day-old infection were fifth-stage preadults, a few had reached sexual maturity, a few were in the fourth molt, and about one fifth were still in the fourth stage. There is little doubt, however, that the majority of infective larvae of this species develop to sexual maturity during the third week of parasitic life, as the egg production peak is normally at-

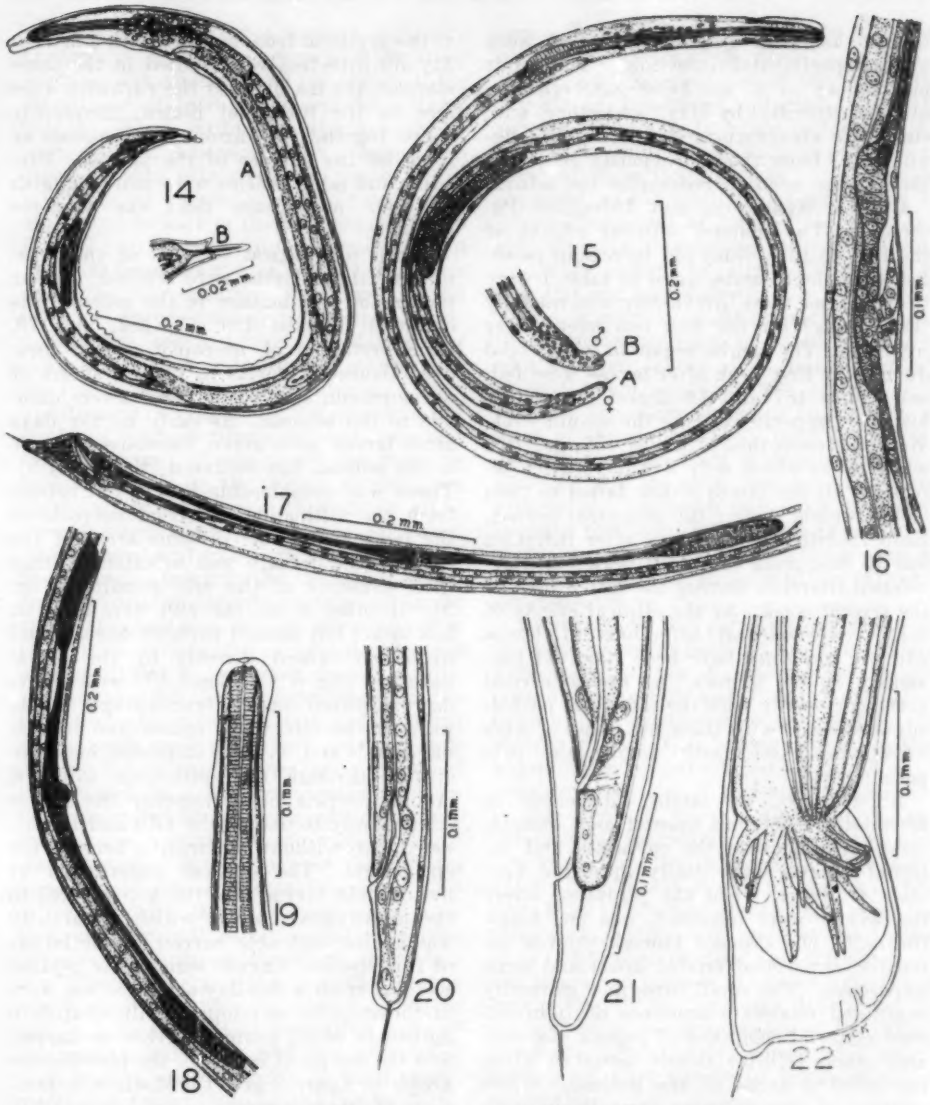

Plate 3—Parasitic development of Nematodirus spathiger.

Fig. 14—Early fourth-stage larva in third molt, five-day infection, (A) entire larva, (B) posterior end.

Fig. 15—Early fourth-stage larva, five-day infection, (A) entire female, (B) posterior end of male.

Fig. 16—Early fourth-stage female, five-day infection, showing further development of reproductive system.

Fig. 17—Late fourth-stage male, eight-day infection, showing reproductive system.

Fig. 18—Late fourth-stage female, eight-day infection, showing reproductive system.

Fig. 19 and 20—Anterior and posterior ends, respectively, of late fourth-stage female, eight-day infection.

Fig. 21 and 22—Posterior ends of young, fifth-stage female and male, respectively, in fourth molt, twelve-day infection.

tained the latter part of the third week after experimental infection.[7] The adult morphology of *N. spathiger* has been adequately described by May [9] and others and, since our observations did not differ significantly from those previously recorded, there is no need to redescribe the adults.

Clinical Symptoms and Intestinal Pathology.—The general clinical effects of the five- to fifteen-day-old infections established in the 6 lambs listed in table 1 were the same as those previously recorded by the writers [7] for the first two weeks after infection. The lambs began to go off feed during the first week after larvae were fed, and lambs 187 and 79 almost completely lost their appetites during the second week. With the exception of lambs 158 and 199, which were killed only five days after infection, all the lambs either failed to gain or lost weight during the prepatent period; lamb 79 killed fifteen days after infection lost 10 lb. during this short period and developed diarrhea during the latter part of the second week. As the clinical effects of heavy experimental infections of lambs with *N. spathiger* have been reported previously by the writers,[7] we are concerned herein primarily with the intestinal pathology associated with the acute phase of such infections, particularly during the prepatent period.

At necropsy, the lambs with eight- to fifteen-day infections showed some emaciation but otherwise the carcasses and internal organs superficially appeared normal. However, when the jejunum, where the worms were localized, was cut longitudinally, the exposed mucosa showed extensive, superficial eroded areas and some hyperemia. The small intestines generally contained moderate amounts of light-colored material composed of ingesta, mucous, and much cellular debris sloughed from the affected areas of the mucosa. When sections of the jejunum from lambs with five- to twelve-day infections were placed in warm 0.85 per cent saline solution a few minutes after death, then opened lengthwise and examined microscopically, relatively few larvae were seen free in the intestinal lumen or on the surface of the mucosa; within a few minutes, however, large numbers of larval worms emerged from the intervillar spaces and moved about with great vigor on the surface of the mucosa. However, when a similar piece of the jejunum from lamb 79 with a fifteen-day-old infection was studied in the same manner, the majority of the parasites were free in the intestinal lumen, frequently coiled together in various sized masses or lying on the surface of the mucosa. Preadult and adult worms were more sluggish in their movements than the younger parasites.

When histological sections of the jejunum of the 6 lambs were studied, a clear picture of the damage to the mucosa was obtained. Lambs 199, 77, 187, and 79, which retained at necropsy several hundred thousand worms in various stages of development, showed the most severe damage to the mucosa. As early as five days after larvae were given, extensive damage to the mucosa had occurred (fig. 8 and 9). There was considerable loss of epithelium from the villi and superficial necrosis of the lamina propria; in some areas of the mucosa, the damage was so extensive that little evidence of the villi remained (fig. 9). In other areas, the villi were more or less intact but showed variable damage and distortion caused directly by the larval parasites (fig. 8). In lamb 199 with a five-day infection many fourth-stage larvae were in the intervillar spaces and intestinal glands and, in some instances, had penetrated through the epithelium into the lamina propria but commonly the larvae seemed only to distort the villi and intestinal glands without penetrating beneath the epithelium. The deepest penetration of five-day-old larvae into the mucosa was to the muscularis mucosa, which apparently was an impenetrable barrier to the larvae of this species. Larvae seen in the jejunal sections from a five-day-old infection were in the stage of development illustrated in figures 14 to 16, young fourth-stage larvae, and the sectioned larvae in the photomicrograph in figure 8 give the distinct impression of their vigorous, lashing motility when alive.

Sections of the jejunum obtained from lamb 77 with an eight-day-old infection (fig. 10 and 11) and from lamb 187 with a twelve-day-old infection showed essentially the same features as those from the five-day-old infection, except the immature parasites were larger in size. Many instances were found where the anterior ends of the larvae had penetrated to the muscularis mucosa (fig. 11), causing it to bulge

slightly, but none were seen to penetrate through it. Extensive necrosis of the villar surfaces was common, and the lumen of the intestine contained much necrotic cellular debris. When living fourth-stage larvae were studied microscopically, various elements of this cellular debris, consisting of remnants of epithelial cells, blood cells, etc., could be seen in their intestines, which showed they ingest cellular as well as fluid material from the gut. Evidence that these parasites may at times feed upon living cells or tissue was not definitely ascertained but seems likely considering the close association of the larval stages with the tissues of the intestinal mucosa.

Although sections of the jejunum of lamb 79 with a fifteen-day-old infection showed tissue damage much the same as that observed in the other lambs, only rarely were immature fifth-stage or adult parasites seen penetrating into the intervillar spaces and intestinal glands, most of the parasites being in the intestinal lumen or at the superficial villar surfaces (fig. 12 and 13). Sections of the jejunum of a lamb with a naturally acquired infection of over 30,000 *N. spathiger*, mainly adults, showed most of the parasites to be in the lumen of the intestine, but a few adult worms were seen with their anterior ends in the intervillar spaces and intestinal glands and, in a few rare instances, they had penetrated beneath the epithelium into the lamina propria of the villi. In this case, however, the damage to the mucosa appeared slight in comparison with that associated with the afore-mentioned massive, experimental infections of immature parasites. It appears, therefore, that when the larvae are growing rapidly in the host's small intestine, they are very active and in intimate contact with the tissues of the mucosa, but when growth is completed they become more sluggish in their movements and tend to inhabit the lumen of the intestine.

As in our previous study,[7] only minor changes of doubtful significance were noted in the blood. Total red and white cell counts, hemoglobin and hematocrit levels, and differential white cell counts were within normal limits during the experimental periods of fifteen days or less.

DISCUSSION

Comparison of Nematodirus Life Cycles.—From the information now available on the life cycles of *N. filicollis, N. helvetianus,* and *N. spathiger* in the respective reports of Boulenger,[1] Herlich,[6] and the present writers, only minor differences are evident in the general pattern of development of these three species. This is to be expected, as the adult males differ only in minor characters of bursae, spicules, and size, and the females are almost indistinguishable except for slight differences in size and egg morphology.

The preparasitic development of these three species appears to be identical, except infective larvae of *N. helvetianus* are about 0.1 mm. longer on the average than those of the other species.[5] Differences in time of development of infective larvae and their release from the egg membranes recorded for the three species in various reports are probably due more to differences in temperature to which egg cultures were exposed and culture methods than to any real difference in pace of preparasitic development.

In regard to the parasitic development, however, there appears to be a significant difference between *N. helvetianus*, as reported by Herlich and Porter[6] and Herlich,[5] and *N. spathiger*, as observed by the writers, in that *N. helvetianus* in calves has a minimum prepatent period of about 3 weeks, whereas for *N. spathiger* in lambs it is only about 2 weeks. Although the developmental morphology of these two species is similar, the third and fourth molts of *N. helvetianus* occur in calves a few days later in the prepatent period than the comparable molts of *N. spathiger* in lambs. It is not known whether this difference in the prepatent periods of the two species is due primarily to intrinsic differences in the species or to the influence of the different hosts involved. No comparable information on other species of Nematodirus is available.

An additional point of interest in regard to the parasitic development of Nematodirus spp. is worthy of mention. May[9] described the great development of the excretory system of adult *N. spathiger*, inferring that other species of the genus were similar in this respect. Briefly, the excretory system consists of an excretory pore and excretory tube located in the posterior esophageal region, which divides into two branches connecting with two

large subventral renette cells (excretory or cervical glands) each with a large nucleus. The renette cells, one extending farther posteriorly than the other, occupy about one fifth the body length. Although the renette cells are prominent structures in the adult nematodes of the genus Nematodirus, no mention was made of such structures in the parasitic larvae of *N. filicollis* by Boulenger[1] or of *N. helvetianus* by Herlich.[5] Certainly in the parasitic development of *N. spathiger* the excretory system is an outstanding morphological feature, which undoubtedly plays a particularly important rôle in larval metabolism, as the renette cells are larger in proportion to body size in the parasitic larvae than in the free-living stages and adults.

In the preparasitic, infective larvae the excretory system is relatively small, consisting mainly of the excretory pore, common duct (the posterior portion is contractile) and two small, pyriform renette cells only about 20 μ long. The entire excretory system, located at the base of the esophagus, is only about one twentieth the length of the infective larvae. In parasitic larvae in the third molt (early fourth stage), however, the excretory system extends almost two thirds the length of the body and in larger fourth-stage larvae may occupy half the body length, the most conspicuous feature at all times being the renette cells. Other species of Nematodirus probably show similar great development of the renette cells during the parasitic stages, although this has not been specifically reported, but similar observations have been made on certain other strongyloid, trichostrongyloid, and metastrongyloid nematodes, which indicate that excretory systems with huge renette cells are probably characteristic of most nematodes of these groups.

Although most workers consider excretion as the primary function of the nematode excretory system, little is known of the chemical nature of the secretions eliminated from the excretory systems of trichostrongyles or most other main groups of parasitic nematodes. However, the possibility that such large excretory systems of certain species of pathogenic nematodes of domestic animals may excrete substances toxic or otherwise injurious to the tissues of the hosts has interesting implications.

Clinical Symptoms and Intestinal Pathology of Infections with Nematodirus spp.—In general, the clinical symptoms associated by various authors with heavy natural or experimental infections of domestic ruminants with various species of Nematodirus are very much the same regardless of differences in species of host or parasite. The common symptoms reported are anorexia, diarrhea, and weight gain retardation or weight loss often to the extent of emaciation. Some reports of outbreaks of parasitic disease in which Nematodirus spp. were primarily involved, such as that of Kingsbury,[6] also showed some mortality loss. There is also agreement in the reports of Herlich and Porter,[6] the writers,[7] and the unpublished work of Kauzal as cited by Gordon,[4] in that the most acute clinical effects, resulting from experimental infections of various species of Nematodirus in lambs and calves, occur during the prepatent period of infection; *i.e.*, when all or the bulk of the Nematodirus population is immature. We believe that immature parasites of this genus are more injurious to their hosts than adults, because immature worms are more intimately associated with the tissues of the intestinal mucosa, are more vigorous in their movements, and produce more severe tissue damage than adult worms. Certain metabolic differences between immature and mature worms, not now known or apparent, may also be involved.

Herlich[5] reported finding, in tissue sections of the small intestine of a calf killed 15 days after being given 87,000 larvae of *N. helvetianus*, immature worms coiled among the villi and degeneration of villar epithelium adjacent to the worms. The writers made similar observations on lambs killed 5, 8, 12, and 15 days after giving large doses of *N. spathiger* larvae to lambs but noted more extensive damage to the intestinal mucosa, such as erosion and necrosis of the villar surfaces and superficial lamina propria, than did Herlich.[5] This, however, may have been due to the fact that Herlich[5] used smaller doses of larvae and a larger host animal than did the writers.

It is of interest that the pathological changes in the small intestine of pigeons resulting from heavy infections of *Ornithostrongylus quadriradiatus*, a trichostrongylid nematode commonly parasitic in this host, as reported by Cuvillier,[3] were simi-

lar in many respects to those observed by the writers in lambs infected with *N. spathiger*.

SUMMARY

1) The life cycle of *Nematodirus spathiger* was essentially similar to those of other species of the genus previously reported, namely, *Nematodirus filicollis* by Boulenger [1] and *Nematodirus helvetianus* by Herlich.[5] However, the minimum prepatent period of *N. spathiger* was about two weeks in lambs compared to about three weeks for *N. helvetianus* in calves; no comparable information is available for *N. filicollis*.

2) During the prepatent period of massive, experimental infections of *N. spathiger* in lambs, many fourth-stage larvae were in intimate contact with the jejunal mucosa. Some larvae penetrated deep into the intestinal glands as far as, but not through, the muscularis mucosa and beneath the epithelium into the lamina propria. As the parasites matured, however, they were found mainly in the lumen of the intestine but, rarely, similar penetration of the mucosa by adult parasites occurred.

3) An outstanding feature of the parasitic larvae of *N. spathiger*, not heretofore reported in other species of this genus but probably present, was the great development of the excretory system, particularly the renette cells, which in early fourth-stage larvae extended two thirds the length of the body.

4) The parasitic larvae, and to some extent adults, of *N. spathiger* caused direct mechanical damage to, and distortion of, the jejunal epithelium and villi. Furthermore, heavy experimental infections in lambs caused directly or indirectly degenerative changes in the jejunal mucosa, consisting of erosion and necrosis of the epithelium and villi and some hyperemia, particularly during the prepatent period.

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The Effect of Transmissible Gastroenteritis on the Metabolism of Baby Pigs

ELWOOD F. REBER, Ph.D., and C. K. WHITEHAIR, D.V.M., Ph.D.

Urbana, Illinois

TRANSMISSIBLE gastroenteritis in pigs was described by Doyle and Hutchings[1] in 1946. In 1948, Whitehair *et al.*[2] reported an endemic digestive disturbance in pigs that was believed similar to this disease. Evidence to date indicates that an infectious agent is the primary cause of this disturbance. The mortality is high when pigs under a week of age are exposed to this infectious agent. Symptoms of vomiting, diarrhea, dehydration, and emaciation are noted especially in pigs older than 1 week. These symptoms suggest that this disturbance should influence the nutritive balances and growth rate of pre- and postweanling pigs. This is a report on the effect of transmissible gastroenteritis infection on body constituents, food consumption, and the retention of water, nitrogen, sodium, and potassium by 26-day-old pigs.

MATERIALS AND METHODS

Two crossbred (Duroc boar x Hampshire sows) litters of 6 female pigs each were used in this experiment. The pigs were selected at 2 days of age from healthy parent stock in a herd having no previous history of digestive disturbances. They were divided into an experimental group and a control group. Three pigs from each litter were used in each group with equal weight distribution. At 26 days of age, each pig in the experimental group was given orally 1 ml. of supernatant from minced lung tissue* suspension.[3] These pigs were maintained in a separate environment and attended by different personnel than were the control pigs.

The pigs were maintained in individual metabolism cages designed for quantitative collection of urine, feces, vomitus, and refused feed. Table 1 presents the composition of the ration fed in the experiment. It was a modification of the semipurified ration used previously by the authors[4] and contained, by analyses, 178 mg. sodium and 741 mg. potassium per 100 Gm. of ration, 5.1 per cent nitrogen and 8.7 per cent moisture. It was a low sodium-high potassium ration, the ratio of potassium to sodium being 4.16.

The ration was fed five times daily as a "synthetic" milk containing 20 per cent total solids. The water and solids were measured immediately before feeding and mixed in a blendor. Except for the time the experimental pigs were sick, each pig was fed approximately the same amount of feed. A 72-hour preliminary collection period was conducted on each pig previous to the time the experimental group was exposed. This collection period is labeled period 1 in the tables, and the figures given are averages for 24-hour periods. Four 24-hour collection periods were conducted on each pig after exposure. The excreta were collected immediately after each feeding and in the case of the experimental pigs the excreta were usually collected immediately after being voided in order to prevent mixing of feces and urine. The excreta, vomitus, and unconsumed feed were kept refrigerated until analyzed. Portions of the ration, excess feed, excreta, and vomitus were analyzed for total solid content by drying to constant weight at 95-100 C. Feed consumption was calculated on the total solids basis. The samples were analyzed for nitrogen content by the Kjeldahl method as described by the Association of Official Agricul-

TABLE 1—Composition of Semipurified Ration*

	Gm.
Vitamin-free casein	35.0
Lard	40.0
Lactose	10.25
Dextrose	10.25
Salts (U.S.P. XIV)	5.0
Vitamin A and D oil (1,000 U.S.P. A., 100 U.S.P. D./Gm.)	0.5
Wheat germ oil (2 I.U. E./Gm.)	1.0

* The following amounts of vitamins were added to 200.0 Gm. of dry ration: thiamin, 1.1 mg.; riboflavin, 1.8 mg.; nicotinic acid, 10.1 mg.; inositol, 26.8 mg.; choline, 260.0 mg.; para-aminobenzoic acid, 5.0 mg.; folic acid, 0.13 mg.; biotin, 0.025 mg.; pyridoxin, 2.0 mg.; calcium pantothenate, 7.1 mg.; ascorbic acid, 130.0 mg.; vitamin B_{12}, 10.0 μg.; menadione, 0.29 mg.

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tural Chemists.[5] Portions of the materials collected were wet ashed with equal parts of sulfuric and nitric acids. This digested material properly diluted was used for the determination of sodium and potassium by flame photometry. Lithium was used as an internal standard.

Blood samples were obtained from the anterior vena cava at the time of infection and at the end of the experiment. Hemoglobin was determined by an acid hematin procedure which had been standardized by the method of Wong.[6] Plasma protein was determined by the copper sulfate specific gravity method as described by Gradwohl.[7] Blood sugar was determined by a modified Folin-Wu method.[8] The flame photometer was used to analyze serum for sodium and potassium content.

Records were maintained on weight and symptoms. Rectal temperatures were taken twice daily. Four days after the pigs were infected, they were destroyed for tissue analyses. The internal organs were weighed and their percentage of body weight calculated.

TABLE 2—The Average Food Consumed and Water, Nitrogen, Sodium, and Potassium Retained by the 6 Noninfected Pigs and the 6 Pigs Infected with Transmissible Gastroenteritis

		Retention			
Period (24-hr.)	Food intake (Gm.)	Water (Gm.)	Nitrogen (Gm.)	Sodium (mg.)	Potassium (mg.)
1*	160	428	6.9	295	758
2	131	417	4.8	201	554
3	92	180	1.4	41	−161
4	88	334	2.7	134	137
5	66	207	1.3	44	26
Ave. (Infected)**	94	285	2.6	105	139
Ave. (Noninfected)†	160	455	6.5	288	693

* The pigs were exposed at the end of period 1. Figures are averages of three preliminary days.
** Average of periods 2 to 5 inclusive.
† Daily figures not shown.

RESULTS

Twenty-four hours after the 6 pigs were exposed 4 of them started to vomit. The onset of this symptom in the 6 pigs varied from twenty-four to seventy-two hours. In the individual pig, the symptom of vomiting persisted for 24 to 48 hours. Vomiting was followed by diarrhea and decreased appetite. The diarrhea continued for four days. The pigs were destroyed four days after they were infected. All the exposed pigs vomited and had diarrhea.

Body Weight.—The initial average weights of the pigs in the control and infected groups were 3,445 and 3,448 Gm., respectively. The diseased pigs lost an average of 4 per cent of their body weight during the last forty-eight hours of the experimental period. During the seven-day period, which included the 72-hour preliminary period for both groups, the noninfected pigs gained 52 per cent and the infected pigs gained 30 per cent of their initial body weight.

Food Consumption. — Average food intakes (total solids) of the 6 infected pigs are shown in table 2. The average food intake was reduced within twenty-four hours after infection and remained smaller through the experiment. The food consumed by the noninfected pigs averaged 160 Gm. per 24-hour period, whereas, the infected pigs consumed an average of 94 Gm. per 24-hour period.

Water Retention.—The average amounts of water retained by the infected pigs for successive periods are presented in table 2. The amount of water retained by the pigs following infection was about one half that retained by the pigs during the preliminary period. The average amounts of water retained by the infected and noninfected pigs for the entire experiment are presented in table 2. Table 3 presents the amount of water excreted in the feces by the infected pigs. The fecal water excreted by the 6 pigs during the preliminary period was found to average 1.5 Gm. per twenty-four hours, while following infection there was a fortyfold increase.

Nitrogen Retention. — The nitrogen retained by the infected pigs for twenty-four-hour periods is shown in table 2. The

TABLE 3—The Average Fecal Water, Nitrogen, Sodium, and Potassium for the 6 Noninfected Pigs and the 6 Pigs Infected with Transmissible Gastroenteritis

		Excretion		
Period (24-hr.)	Water (Gm.)	Nitrogen (Gm.)	Sodium (mg.)	Potassium (mg.)
1*	1.5	0.03	1.3	11
2	1.3	0.10	3.5	27
3	63.5	1.0[illegible]	78.5	256
4	37.5	0.53	32.0	272
5	60.5	0.61	85.2	369
Ave. (Infected)**	40.7	0.56	49.8	231
Ave. (Noninfected)†	1.3	0.03	1.1	5

* The pigs were exposed at the end of period 1. Figures given are averages for three preliminary days.
** Average for periods 2 to 5 inclusive.
† Daily averages not shown.

nitrogen retention by the infected pigs followed the same general pattern as to their ability to retain water. The nitrogen retained by the infected pigs after period 2 was about one third that which they retained during the preliminary period. The urinary nitrogen of the infected pigs was approximately 70 per cent of the urinary nitrogen of the noninfected pigs. Table 3 presents the fecal nitrogen for successive 24-hour periods. The fecal nitrogen of the infected pigs was about fifteen times greater than the amount excreted by these pigs during the preliminary period.

TABLE 4—The Average Levels of Blood Constituents of 6 Noninfected Pigs and of 6 Pigs Infected with Transmissible Gastroenteritis

Constituent	Noninfected	Infected
Hemoglobin (Gm./100 cc.)	10.6 (30)*	13.0 (6)
Plasma protein (Gm./100 cc.)	5.9 (30)	5.8 (6)
Blood glucose (mg./100 cc.)	90.0 (18)	63.0 (6)
Serum sodium (mEq./liter)	134.0 (23)	134.0 (6)
Serum potassium (mEq./liter)	9.2 (5)	8.6 (6)

* Number in parentheses represents the number of blood samples.

Electrolyte Retention.—The average amounts of sodium and potassium retained by the 6 infected pigs for the successive 24-hour periods are presented in table 2. There was a considerable decrease in the sodium and potassium retained by the infected pigs. The decrease was greatest during the third and fifth periods with a negative potassium balance for the third period. This was the only constituent of the four measured which showed an average negative balance for a period. During the time the individual pigs showed a negative potassium balance, the feces were high in potassium content and this was usually accompanied by a decreased urinary potassium. The incidence of increased fecal potassium had a closer correlation with negative potassium balance than did the incidence of vomition. The average amounts of sodium and potassium retained by the 6 infected and 6 noninfected pigs are presented in table 2.

The levels of blood constituents of infected and noninfected pigs are presented in table 4. Transmissible gastroenteritis infection did not influence the average body temperature, plasma protein level, or the serum sodium level. The infection did cause a decrease in the average blood glucose and a slight decrease in serum potassium while the hemoglobin value was increased.

Postmortem lesions observed were catarrhal gastritis and enteritis. The intestinal contents were yellow and of foamy consistency with a foul odor. The organ weights of all the pigs were obtained and their percentage of body weight calculated. When compared on the basis of percentage of body weight there were no differences between the average values of the 6 infected and 6 noninfected pigs with respect to the weight of heart, liver, spleen, kidney, or empty large and small intestine either before or after drying the latter to constant weight at 95-100 C.

DISCUSSION

The food consumed by the infected pigs was approximately 60 per cent of the amount consumed by the noninfected pigs. The retention of water was of the same magnitude. The infected pigs retained approximately 60 per cent of the amount of water retained by the noninfected pigs. The estimation of water balance is affected by the loss of water due to evaporation during the collection and storage periods. The amount offered to each pig was accurately known, but the amount of water refused or excreted in every case would be larger than the figures indicate. There was a greater variation in water retention than in food consumption by the individual infected pigs. Water retention appeared to be related to the length of incubation time and the duration of diarrhea for each pig. The data in tables 2 and 5 show the infection

TABLE 5—The Percentage of Food Consumed and the Percentage of Nitrogen, Sodium, and Potassium Intake which Was Retained by the 6 Noninfected Pigs and the 6 Pigs Infected with Transmissible Gastroenteritis

		Retained		
Period (24-hr.)	Food consumed (Gm.)	Nitrogen (Gm.)	Sodium (mg.)	Potassium (mg.)
1*	100	77	95	58
2	82	66	79	52
3	58	27	23	−22
4	55	55	78	19
5	41	34	33	5
Ave. (Infected)**	59	43	50	14
Ave. (Noninfected)†	100	73	92	53

* The pigs were exposed at the end of period 1. Figures given are averages for three preliminary days.
** Average for periods 2 to 5 inclusive.
† Daily averages not shown.

caused a decreased food consumption, accompanied by a decreased retention of water, nitrogen, sodium, and potassium. The decreased amounts of nutrients retained was not due to the decreased food consumption alone but was also due to a decreased efficiency of retention. The percentage of the nitrogen, sodium, and potassium intakes which were retained by the infected and noninfected pigs are presented in table 5. Part of the decrease in nitrogen retention was due to vomiting; however, the major portion of the decrease was due to decreased food consumption, increased fecal nitrogen, and proportionately increased urinary nitrogen. The decreased sodium and potassium retentions were due to increased fecal excretion and loss of the cations due to vomiting.

The average daily intake of sodium by the noninfected pigs was 312 mg. and the average amount retained was 92 per cent. This occurred on a low level of dietary sodium. The potassium in the ration was 4.16 times greater than the sodium in the ration. This high average daily intake of potassium by the noninfected pigs was 1,297 mg. and the average amount retained was 53 per cent.

The infection of the pigs with transmissible gastroenteritis resulted in a decreased food consumption and, in addition, an average of 50 per cent of the sodium and 14 per cent of the potassium was retained.

The high dietary level and intake level of potassium by the infected pigs was not adequate to prevent an average negative potassium balance. Therefore, these data indicate that the mechanisms for absorbing and retaining sodium by the pig are not disturbed to the extent that mechanisms for absorption and retention of potassium are disturbed by infection with transmissible gastroenteritis. These data indicate that, of those nutrients studied, potassium should be given primary attention in an attempt to overcome the effects of transmissible gastroenteritis infection upon the nutrition of the baby pig.

SUMMARY

The effect of transmissible gastroenteritis infection upon feed consumption, water, nitrogen, sodium, and potassium balances, and blood constituents of 6 young pigs was investigated. Six pigs were maintained as noninfected controls. The pigs were 26 days of age at the time of infection. The incubation period as measured by the appearance of vomition or diarrhea varied from twenty-four to seventy-two hours. All the infected pigs showed symptoms of the disease. The pigs lost an average of 4 per cent of their body weight during the last two 24-hour periods. Feed consumption, weight gain, blood glucose, and the amount of water, nitrogen, sodium, and potassium retained were decreased by the infection. Following infection the fecal water was increased forty-fold and hemoglobin values were slightly increased. There was no elevation in the average temperature of the exposed pigs. The heart, liver, kidney, spleen, and intestine weights were calculated as percentage of body weight and comparison of these values for the infected with noninfected pigs revealed no differences.

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Fragility of Erythrocytes in Blood from Swine of Two Age Groups

A. EDWARD A. HUDSON, Ph.D.

Raleigh, North Carolina

THE HIGH death rate among suckling pigs during their first few days of life presents a challenge to animal industry and to veterinary medicine. The causes of many of these deaths will remain unexplained until more is known of the normal changes taking place in the healthy animal and until further information is gained about the pathological changes occurring in the pig suffering from the less common diseases to which swine are susceptible. A step toward the determination of the afore-mentioned changes should include a complete hematological picture.

The inadequacy of information on normal blood values and changes brought about in the circulating blood of swine is evidenced by gaps in the published data.[1] The present study, which was a part of a more comprehensive investigation of the hematology of normal swine, was conducted to aid in filling the gap in current knowledge relating to the fragility of the erythrocyte of the pig.

PROCEDURE

Resistance of erythrocytes to the hemolytic action of graded concentrations of hypotonic saline solutions was used as an index of red cell fragility. A modification of the technique described by Rappaport[2] was adopted. One drop of blood was added to each of a series of small tubes containing 1 ml. of saline of increasing concentrations, in increments of 0.05 per cent, from 0.30 to 0.85 per cent of sodium chloride. After the tubes had stood two hours at room temperature, they were centrifuged. Initial hemolysis (minimum resistance) was recognized by the first faint trace of redness of the supernatant fluid; complete hemolysis (maximum resistance) was indicated by the absence of red cells in the bottom of the tube. Degrees of hemolysis between initial and complete were estimated by noting the extent of redness of the supernatant fluid and the amount of the erythrocytes remaining intact.

It was assumed that the hematological picture of the baby pig is in a transitory state and that the blood of pigs approaching maturity is in a state of equilibrium. Thus, for comparative purposes 20 suckling pigs (5 litter mates from each of 4 litters) not over 72 hours of age and 20 pigs approximately 7 months of age were used as sources of blood in this study. The pigs were selected by random sampling from a farm herd that was free of clinical abnormalities.

RESULTS

The means of the observations on the blood samples from the two age groups are shown in figure 1. Thirteen of the 20 samples of baby pig blood underwent initial hemolysis in a concentration of 0.80 per cent saline. All the samples from this group showed some degree of hemolysis in the 0.75 per cent saline concentration. The erythrocytes of 5 suckling pigs, 3 from one litter and 2 from another, were completely hemolyzed at a saline concentration of 0.55 per cent; of 10 additional pigs, hemolysis was complete at the 0.50 per cent concentration; and of the remaining 5, hemolysis was complete at a concentration of 0.45 per cent.

In the 7-month-old pigs, hemolysis had begun in three of the specimens at a concentration of 0.80 per cent. Initial hemolysis had taken place in all but three of the samples under observation at a concentration of 0.75 per cent. Comp. ·e hemolysis had occurred in all of the san. ples in this age group at a 0.45 per cent concentration of saline.

No difference in erythrocyte fragility in the sexes was apparent.

DISCUSSION

The erythrocyte fragility values obtained in the 7-month age group agree well with the findings reported by Lyon[3] and Rywosch.[4,5] Lyon observed that initial hemolysis of the red cell of pig blood took place at a saline concentration of 0.70 per cent. Both of these workers reported that the erythrocytes of swine examined by them underwent complete hemolysis in a saline concentration of 0.45 per cent.

The erythrocytes from the 72-hour age group were more fragile than those either

From the Animal Nutrition Section, Department of Animal Industry, North Carolina Agricultural Experiment Station, Raleigh.

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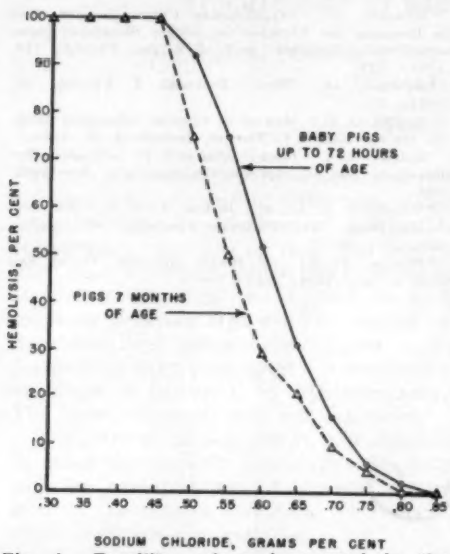

Fig. 1 — Fragility values (expressed in the terms of percentage of erythrocytes that underwent complete hemolysis in graded concentrations of NaCl solution) for pigs in two age groups.

from the 7-month age group or from the swine reported by Lyon [3] and Rywosch.[4,5] These observations add credence to the assumption that the hematological picture of the baby pig is in a transitory state, whereas that of 7-month-old swine is in a state of equilibrium.

Since the most fragile erythrocytes in the normal human subject undergo initial hemolysis at a saline concentration of 0.40 to 0.46 per cent and complete hemolysis at 0.30 to 0.36 per cent,[6,7] apparently the red cell of the pig is more fragile than that of man. Moreover, the normal erythrocyte of the newborn pig is inclined to be more fragile than that of the older pig.

One of the main factors leading to hemolysis is structural weakness of the cell. Changes in the corpuscle membrane, whether congenital or acquired, lead to premature senility of the erythrocyte, thereby bringing about their early destruction. The most common indicator of cell abnormality is spherocytosis.[6] When this condition becomes marked, there is a parallel increase in the fragility of the erythrocytes. Occasional spherocytes were observed in the blood smears of these suckling pigs, but none of the globular cells was detected in the older age groups. The number of observations, however, was insufficient to relate the number of spherocytes to the fragility of the erythrocytes as reported for man.[8]

The destruction of erythrocytes is a continuous process within the normal animal body. In the human subject under certain pathological conditions, this destruction is intensified, often bringing about a marked anemia. Since excessive hemolysis is traced to increased fragility of the red cells (as in hemolytic jaundice) or to the presence of an hemolytic agent, the fragility test has proved to be of great value in differential diagnosis of hemolytic jaundice.[8] Though there is no evidence that the pig is susceptible either to a form of congenital or familial hemolytic jaundice or to hemolytic diseases encountered in the newborn human infant, from time to time it has been observed that baby pigs have been afflicted with a generalized edema and marked hemolysis. Some of these pigs have been stillborn, while others have died a few days after birth. The appearance of these animals resembled cases of hydrops fetalis in the human infant. Other baby pigs, which showed a marked reduction in their hemoglobin levels at birth, expired five days later. Autopsy revealed jaundice, with a marked enlargement of the liver and spleen, suggestive of icterus gravis.

In the studies of causes and prevention of the high mortality of newborn pigs consideration should, therefore, be given to the possibility that the pig is susceptible to some of the hemolytic disturbances encountered in the human infant. In erythroblastosis or hemolytic disease of the newborn human infant, if born alive, the baby may exhibit icterus gravis neonatorum and/or hemolytic anemia.[9] It is possible that abnormal antibody production in the pig during uterogestation may bring about the formation of hemolysable erythrocytes in the newborn.

SUMMARY

Fragility of the erythrocytes of blood from pigs less than 72 hours of age was greater than in swine 7 months of age. These comparative results in normal healthy swine suggest that the hematological picture of the baby pig is in a transitory state.

The determination of erythrocyte fragility is considered as a possible diagnostic measure in determining the causes of some of the losses of baby pigs.

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Procurement of Baby Pigs by Hysterectomy

GEORGE A. YOUNG, D.V.M.; NORMAN R. UNDERDAHL, M.S.;
RONALD W. HINZ, B.S.

Austin, Minnesota

PREVIOUS communications have reported the successful rearing of baby pigs in isolation without the feeding of antibiotics, colostrum, or immune serums. The first report dealt with pigs which were caught in sterile canvas bags and housed in open cages in isolation rooms.[1] The second report described individual isolation units for rearing baby pigs caught in sterile canvas bags or delivered by hysterectomy.[2] The operation itself was not described.

The purpose of this report is to describe in detail the methods used to procure baby pigs by hysterectomy. A general discussion of the results and areas of research applicability is also included.

METHODS

Brood Stock.—The brood sows and gilts have been taken from either a closed Chester White herd, or from a herd of miniature swine being developed at the Institute for experimental purposes. These dams are pen-mated so that a reliable estimate of term is possible. All dams are slaughtered for removal of their pigs from two to six days before expected parturition.

Anaesthesia.—Carbon dioxide anaesthesia before slaughter has been authorized by the U.S.D.A. for the routine commercial killing of hogs. It is also used prior to hysterectomy, which permits salvaging of the carcass after removal of the baby pigs.

The dam from which pigs are to be removed is hoisted by her hind legs into the operative position (fig. 1). Her belly is thoroughly cleansed with soap and water before she is lowered head first into a 55-gallon open-top steel drum filled with CO_2 gas. The dam is allowed to inhale CO_2 for one minute, after which time the barrel is removed, and an abdominal incision is made immediately.

Hysterectomy.—The incision for removal of the gravid uterus is made with a clean sterile butcher knife through the abdominal wall on the midline while the dam is suspended by the hind legs. The initial 3 to 4 inches of the incision is made into the peritoneal cavity just cephalad to a point opposite the posterior-most teats. A rubber knife-guard held by the left hand is then forced into the peritoneal cavity and the point of the butcher knife is inserted into the guard which is held in the palm of the operator's surgically clean left hand. The incision is then extended 12-15 inches cephalad while forcing the gravid uterus and other viscera aside with the back of the left hand. The operator, by reaching into the cavity, can then lift the horns of the gravid uterus up to and out of the incision (fig. 2). After the entire uterus is outside the sow it is cut free, by excising through the cervix (fig. 3), and is passed quickly through an antiseptic lock into a hooded work table where the pigs are liberated.

Two men, with surgically clean hands, working through canvas sleeves, tear the pigs from the uterus as soon as it enters the hood. This is done by placing both hands over the pig, grasping the uterus firmly, and tearing it by rotating the wrists and forearms outward (fig. 4). Each pig is rolled free, and then placed aside in a dry area where another operator applies a small crocodile radio clamp to the navel about one inch from the body. After all pigs are freed and all navels are clamped,

Fig. 1—Gilt in process of being anaesthetized with carbon dioxide gas for hysterectomy; operator simultaneously cleans surgical area.

From the Hormel Institute, University of Minnesota, Austin; Dr. Young, associate professor in Veterinary Medicine, Mr. Underdahl and Mr. Hinz, assistant scientists.

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the operator cheeks the nostrils and mouth of each pig, wiping off mucus and membranes with a dry towel wherever necessary. The umbilical cords are then torn midway between the uterus and the pig, and the pigs are wiped dry with sterile laundered feed sacks (fig. 5). Their navels are ligated near the body with light cotton cord, cut so as to leave a stub about an inch long, and are cauterized by swabbing with tincture of iodine. All pigs are weighed, measured for crown-rump length, identified, placed in an enclosed carrying case (fig. 6) and returned to the laboratory where they are housed in individual isolation units (fig. 7 and 8).

Operation Hood.—The covered work table or hood, within which the pigs are removed from the uterus, is illustrated schematically in fig. 9. The table is kept under slight positive air pressure by the constant introduction of warm filtered air. The working temperature of the hood, 100-110 F., is maintained by passing the incoming air through a hot water radiator (fig. 9, items 6, 7, 8). The liquid lock of moderately antiseptic solution serves to introduce the gravid uterus into the hood without creating air currents which might introduce infectious agents.

The filter mediums are ordinary furnace filters followed in series by a rectangular 24 by 24-inch No. 50-FG spun glass wool filterdown pad * to remove possibly infectious materials from the air introduced into the hood. The interior is sterilized before each day of use with formaldehyde gas produced by mixing potassium permanganate and formalin. Poured agar plates are exposed to the air within the hood during operations to check on filter efficiency.

Canvas sleeves (fig. 9-4) are provided for the arms of the operators. The sleeves are sterilized by steam and fixed in place before the hood is gassed. By using plastic and canvas covers, it is possible to set up and gas the hood the day before it is used. To expel the residual formaldehyde gas, and bring the hood up to operation temperature, the blower is started an hour before the hood is to be used.

Inside the hood, an area is available for a carrying case (fig. 9-3) for transporting pigs to the laboratory isolation facilities. The case is sterilized separately inside a canvas bag, and is uncovered only after introduction to the hood. It is provided with a filter of the same material as used in the hood to assure that the air inhaled by the pigs is not contaminated with infectious agents.

Allantoic and amnionic fluids are eliminated from the hood through a perforated plate, at the low point of the work table, into a closed waste receptacle (fig. 9-2). The plate is removable so that the uterus and placental tissues can be discarded into the waste receptacle after liberation of the pigs.

Transfer of Pigs.—After the pigs are transported to the laboratory, in the carrying case, they are transferred to the isolation units (fig.

* American Air Filter Company, Louisville, Ky.

Fig. 2—Pigs encased in utero are lifted up and out of abdomen.

Fig. 3—Uterus with encased pigs is cut free from the dam near the cervix.

Fig. 4—Technician tearing pigs from the uterus (background) and clamping navels (foreground).

Fig. 5—Newborn pig is wiped to remove blood, amnionic tissues, and mucus.

7, 8) in clean, previously gassed (formaldehyde) rooms by attendants wearing sterile coveralls and face masks. The transfer is made quickly to minimize respiratory exposure. Attendants routinely dampen their hands with a mild antiseptic solution (quaterinary ammonium compound) when handling the pigs. The rooms and isolation units are kept at 95-100 F. for the first few days and then may be lowered to 85 F.

Diet for Pigs.—The diets previously reported[1, 2] or several modifications of them may be used, depending on the objective for use of the pigs. Several suggestions appropriate for those interested in application of the techniques described are indicated below.

A diet containing one whole egg per quart of milk fed with mineral continuously to 4 weeks of age has been satisfactory for growth experiments. Increasing the protein by addition of egg has proved more satisfactory than increasing fat by the addition of cream, as reported earlier,[2] and is also less expensive.

Disposition of the Dam.—Immediately after hysterectomy, the dam is processed for food in the following manner.

1) The operator bleeds the dam in the usual manner immediately after removal of the uterus.

2) The skin around the incision is undercut approximately 2 inches in every direction (fig. 10) in order to separate it from the subcutaneous fat to form a free skin flap for use in closing the abdominal opening.

3) The inner surfaces of the skin flaps are stapled together (Bostitch P 4-8 stapling plier with ½-inch staple) in a zig-zag manner with enough overlapping to effect a water-tight seal of the incision (fig. 11).

4) A continuous shoestring-type suture is placed proximal to the staples, using heavy cotton cord inserted with a bagging needle (fig. 12). The dam is lowered to the floor, and while on her back, the suture is drawn so tight it is almost buried in the skin.

5) Rosin is applied over the entire incision.

6) The dam is placed on a packing plant conveyor line and processed in the usual manner. The suture must be tight enough to exclude scalding tank water, and strong enough to remain closed during the rugged process of dehairing (polisher).

Fig. 6—Technicians removing carrying case from liberation hood. Pigs are transported to isolation quarters in the filter-equipped case.

RESULTS AND DISCUSSION

General.—The concept of obtaining pigs by means of hysterectomy for experimental purposes originated in May, 1952. Since that time, a practical and relatively simple method has been worked out at the Institute. The technique used is sufficiently precise to insure that newborn pigs will survive in the absence of infection. Current mortalities among Chester White pigs (table 1) are less than 5 per cent.

TABLE 1—Mortality Among Pigs Farrowed by Hysterectomy and Housed in Individual Isolation Units (January through May, 1954)

Breed	No. started	No. died	Mortality (%)
Chester White	162	7	4.4
Miniature*	127	16	12.7

* A line of miniature swine is being developed at the Institute primarily for an experimental animal. Project is under the supervision of David C. England, Ph.D.

Losses among miniature swine thus far have been considerably greater than among newborn Chester White pigs. Several factors have contributed to the difference. The equipment was originally designed for Chester White pigs. Miniature pigs, because they are from one third to two thirds the size of Chester White pigs at birth, have a greater requirement for additional heat.[3] The losses, most of which occurred in January, have been considerably lower since the greater heat requirements have been met. The isolation units are too large, and the feeding trays too deep for the miniature pigs to perform to best advantage.

Stillborn pigs have been rare in litters delivered by hysterectomy. Those which were stillborn showed a definite brown discoloration indicating necrosis. A number of weak pigs have been delivered which would have had little likelihood of surviving natural birth. Oxygen has not been necessary to assure survival of any pigs delivered. The CO_2 gas used to anesthetize the sows has acted as a respiratory stimu-

lant to the pigs so that their first breathing is deep and vigorous. A normal breathing pattern developed within a few minutes.

EXPERIMENTAL USES

The hysterectomy technique was developed to provide a relatively convenient and continual supply of disease-free, antibody-devoid baby pigs with which to study virus diseases. It replaced the tedious and variable procurement of pigs by catching them in a sterile canvas bag at natural farrowing. The technique also makes it possible to provide such pigs to other laboratories. Eighteen newborn pigs have been taken by car 100 miles in an enclosed carrying case, with no losses. In the course of researches with pigs obtained by hysterectomy, a number of other uses have become apparent, some of which are indicated.

Disease Experiments.—Disease-free, antibody-devoid baby pigs have been used primarily in the study of swine diseases such as transmissible gastroenteritis, hog cholera, influenza, and a disease suspected to be virus pneumonia of pigs. These pigs have also proved useful in research on human diseases including poliomyelitis, hepatitis, and infant diarrhea.

Pigs obtained by hysterectomy make ideal hosts for experimental transmission of swine diseases. Inoculations may be made within hours of delivery of pigs three to six days premature. Absence of antibody also offers increased opportunity

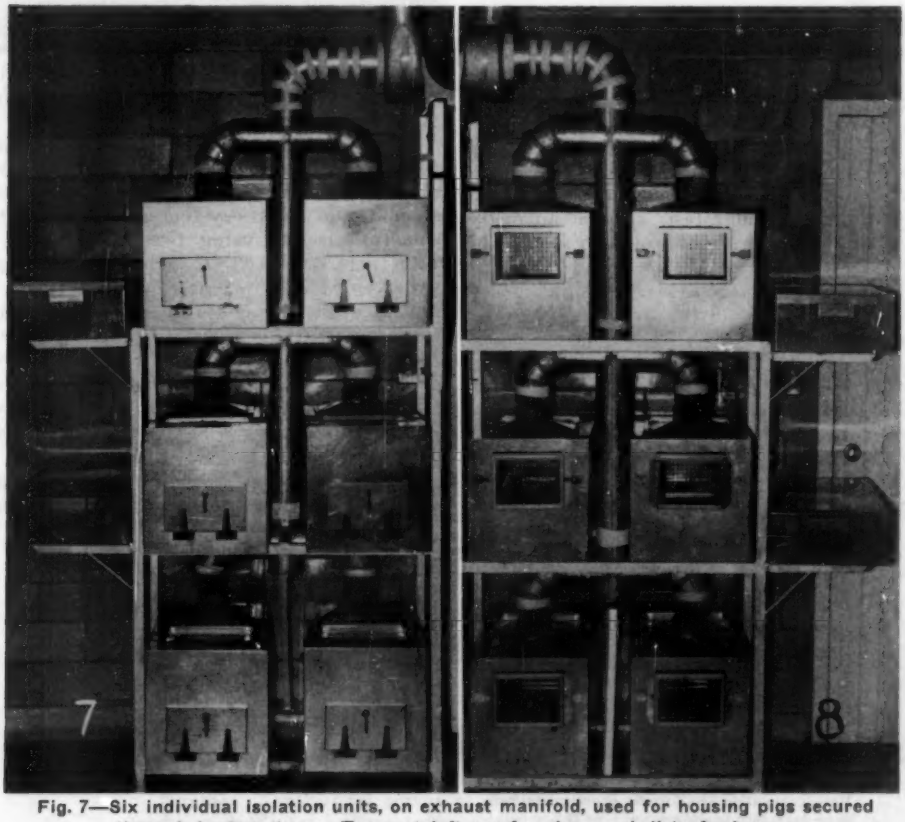

Fig. 7—Six individual isolation units, on exhaust manifold, used for housing pigs secured through hysterectomy. Trays at left are for clean and dirty feed pans.
Fig. 8—Rear view of isolation units showing observation windows.

for infectious agents to become established in the host. A simple host-parasite relationship exists because other agents are not given an opportunity to complicate the experimental disease. In experiments where

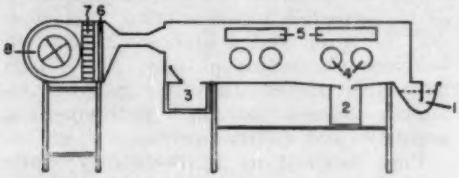

Fig. 9—Liberation hood into which the uterus is placed after hysterectomy for removal of the pigs: (1) antiseptic lock, (2) waste receptacle, (3) carrying case, (4) arm ports for canvas sleeves, (5) observation windows, (6) air filter, (7) hot water radiator, (8) blower.

some host immunity is desirable, specific antibodies may be given orally by stomach tube the first six to eight hours. After that time, absorption of antibody through the intestinal wall is inefficient or impossible.

Insufficient work has been done with poliomyelitis to define whether or not infection occurs in young pigs. Although no symptoms are manifest, specific antibody titers of 1:640 were obtained in serums of young pigs for all three types of virus. Older swine raised in isolation and inoculated four times with virus in adjuvant resulted in serum neutralizing antibody titers of 1:10,000 for types 1 and 2, and 1:2,560 for type 3.[4] Since *gamma* globulin may be prepared from swine serums,[5] swine reared in a controlled environment and injected with all three types of poliomyelitis virus might be used as a source of high titer antibody for injection of children during poliomyelitis epidemics. Despeciation of the protein would be desirable, or perhaps even necessary.

Reports on use of disease-free, antibody-devoid pigs for human infectious and serum hepatitis have indicated inability to establish these viruses in pigs.[6] However, the infectious hepatitis materials used for inoculation had not been previously established as containing virus. Subsequent experiments using serums which contained virus as shown by inoculation of human volunteers have indicated primary infectivity of human infectious hepatitis virus for young pigs devoid of antibody.[7]

Materials from four different sources representing 14 patients with infantile diarrhea have been given 5-day-old pigs to test for transmissible agents to which baby pigs might be susceptible.[7] No transmissible agents were found under the conditions of the test; a variety of material should be tested in newborn antibody-devoid pigs, however, to give maximum opportunity for an agent to become established.

Tissue Culture.—The counterpart of the disease-free, antibody-devoid pig can be obtained in tissue culture. A homologous swine tissue culture system is obtained with relative ease by using tissues from newborn pigs delivered by hysterectomy, pig embryonic tissue extracts, and antibody-devoid newborn pig serum. Nasal mucosa, lung, intestine, liver, spleen, kidney, and testicle have been grown in this laboratory in primary culture on pyrex glass by a roller tube method. Tissues grow rapidly and are useful for study of virus diseases to which swine are susceptible.

Nutrition Experiments.—Studies of the nutritional requirements of baby pigs are

TABLE 2—Comparison of Performance of Pigs Farrowed and Suckled by the Sow with that of Pigs Taken by Hysterectomy

Environment and conditions	No. pigs	Weight at 56 days	Weight at 154 days	Gain lb./day	Feed/cwt. gain
Pigs farrowed by dam, nursed 56 days, and housed to 154 days in a clean, well-ventilated, and temperature-controlled barn with tile pens (ave. 1 year)	225	29	132	1.1	421
Pigs by hysterectomy*					
Brooder isolation first 4 weeks; conditions same as above after that time (ave. 1 year)	115	24	166	1.5	321
Brooder isolation first 4 weeks, experimental barn to 56 days, then to clean drylot (late spring only)	38	27	160	1.4	**

* No colostrum, serum, or antibiotic fed.
** It was not possible to determine feed consumed under system of management used.

Fig. 10 — Salvaging dam after hysterectomy. Skin is undercut to obtain a flap for closure of the abdominal incision.

often complicated by manifestation of diarrhea. Young pigs are easily upset by dietary changes but it is not possible to distinguish between diarrheas caused by dietary inadequacies and those caused by disease. By use of pigs obtained by hysterectomy, disease is controlled so that diarrheas from dietary causes can be evaluated.

Genetic Experiments.—One difficulty in genetic experiments is control of environment so that comparison of performance from litter to litter and from year to year may be more valid. Swine diseases frequently void or adversely affect results by masking actual growth potential of the individual pig. Differences in ability to perform occur between animals in the same breed and even within the same litter. Greatest selection pressure is exerted under standard conditions so that the measured differences are influenced less by environment or nutrition.

A standard environment is possible by using pigs secured through the hysterectomy, reared in brooders, and adapted to ordinary swine-rearing facilities. Practical isolation from swine diseases is not difficult and can be carried out for individual or litter tests with pigs for feed efficiency and rate of growth. Most important—pigs cleansed by hysterectomy must be kept sep-

Fig. 11—Salvaging dam after hysterectomy. Inner surfaces of skin flap are stapled together. Residual fat serves as a cushioned waterproof seal.

Fig. 12—Salvaging dam after hysterectomy. Purse-string suture of heavy cotton cord is used to protect stapled incision from polisher (dehairer) teeth. Sutures are pulled tight so that teeth can not catch the suture cord.

arate from ordinary swine no matter how healthy the latter may appear.

Disease Control and Eradication.—The uses listed above for pigs obtained by hysterectomy are largely experimental. A practical application of the techniques and principles of rearing pigs set forth above is in ridding brood stocks of troublesome diseases. A comparison of performance of two groups of swine from the same basic stock under similar conditions is presented in table 2. Performance of pigs farrowed by dams and nursed by them is compared to performance of pigs farrowed by hysterectomy, reared in brooders for the first four weeks, and then reared under the same type of management as the naturally farrowed swine.

Two obvious advantages of performance by stock farrowed by hysterectomy are the 0.4 lb. per day more rapid growth, and the requirement of 100 lb. less feed for 100 lb. of comparable gain. No antibiotics were fed to the pigs by hysterectomy whereas the naturally farrowed pigs had antibiotics until approximately 100 lb. in weight.

One permissible conclusion is that rigid control of disease makes it possible for the pigs to utilize their feed more efficiently because they are not held back by parasites, bacteria, or viruses. The herd under study has long been plagued by a number of diseases.[8-10] Swine dysentery, transmissible gastroenteritis, and mange have been by-passed by use of the hysterectomy techniques. Applicability of the techniques to other diseases will be determined as herds with other diseases become available for study.

A general misconception about pigs raised in brooders is that they are of the hothouse variety. It should be pointed out that, although care is taken to prevent contact with other swine and exposure to swine diseases, a gradual adaptation is made from the brooder environment to an environment that is practical under good farm management, including clean drylot or pasture. Pigs 4 weeks old or older have little difficulty in adjusting to normal soil bacteria and farm conditions. This is indicated by performance of such pigs in drylot (table 2). No pigs became ill or were lost.

It should be kept in mind that while pigs taken by hysterectomy and housed in isolation units are referred to as disease-free, they are not germ-free. After the first twenty-four hours, they are given pasteurized milk which does have a moderate bacterial flora. This flora likely is beneficial rather than harmful as the bacteria may help the pig to utilize his food and make vitamins for him. Further, as the pig is moved from the isolation units to environments with less strict disease control, he more logically is a pig with minimal disease rather than being disease-free. The important consideration is that parasites, bacteria, viruses, et cetera, which make the pig less efficient as a meat-producing animal are excluded from his environment.

The equipment described in this paper was designed for experimental use in a moderately large swine research program. It is more complex than would be required to clean up outstanding brood stocks for the good swine producer, purebred or commercial. The isolation units would be desirable, but a hood for removal of the pigs to prevent exposure to disease need not be as elaborate as the one described. Hoerlein [11] has devised a relatively inexpensive and simple hood which would fit the requirements of the practitioner. The sow is anesthetized using dry ice in a large garbage can. Pigs may be transported in a covered cardboard box which has been previously gassed with formaldehyde. The dam's carcass could be disposed of through a cooperative local slaughterhouse or locker plant. Performance of the operation in such an establishment is also a possibility.

To those who would like to use the hysterectomy process to eliminate or bypass disease in good breeding lines of swine, the limitations of the placenta as a disease barrier must be considered. The five-layered swine placenta is not fully formed until thirty days, and infectious agents can pass the incomplete barrier to remain in the fetal pig tissues.[12,13] Such a pig would be a source of new infections for pigs he would later contact. Likewise, pigs should not be taken from dams which are in active stages of infection. During bacteremia or viremia, maternal blood would serve as a source of contamination for the pigs.

It should also be emphasized that in the use of these techniques to eradicate infectious diseases from swine, there is no such thing as relatively clean procedures. The

break with infection must be absolute, because of the ability of infectious agents to reproduce themselves. Failure to handle one animal properly results in disease in that animal and possibly in all other animals with which it comes in contact. This note of caution is injected because several instances have been called to the author's attention where poor livability of pigs was encountered due to failure to avoid early exposure of pigs to agents common to their dam's environment.

SUMMARY

A method is described by which baby pigs are delivered before term and reared during the early part of their lives in individual isolation units on modified cow's milk. No colostrum or antibiotics are fed. Livability of pigs is high.

Pigs are obtained from their dam two to six days before expected parturition through removal of the uterus and encased pigs. Pigs are liberated from the uterus within a sterile hood. The dam which was anesthetized with carbon dioxide gas during the operation is bled, and processed for food.

Experimental and applied uses of the techniques are discussed.

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Salmonellosis in Poultry and Poultry Processing Plants in Florida

MILDRED M. GALTON, Sc.M.; DON C. MACKEL, M.S.; A. L. LEWIS, D.V.M.; W. C. HAIRE, D.V.M.; A. V. HARDY, M.D., Dr. P.H.

Jacksonville, Florida

SALMONELLA infections of fowl have aroused much concern during the past decade. They are now recognized as both an economic and a public health problem.

As a part of broad studies of salmonellosis in Florida, diseased poultry, pullorum reactors, and apparently normal birds have been examined for evidence of Salmonella infection. Following the frequent recovery of Salmonella from human and animal food products and from the environment of large abattoirs slaughtering swine, similar studies in local poultry processing plants were undertaken. This report provides bacteriological findings on fowl obtained during a five-year period and data on the prevalence of Salmonella in the environment of four poultry processing plants.

During the period Jan. 1, 1949, to Dec. 31, 1953, 718 chickens or turkeys were examined postmortem and were studied by multiple bacteriological tests. Of these, 155 had been tested with *pullorum* antigens by the Florida Livestock Board and reacted to the whole blood plate test, 434 were submitted by veterinarians or flock owners for examination to determine the cause of illness or death, and 129 presumably normal birds were examined at the processing plants. Cultures were made of portions of the heart, liver, spleen, gall bladder, oviduct or ovary, or both, of any cysts observed, and of the cecal contents. In removing the various organs, precautions to avoid the contamination of one organ by the contents of another were observed. The materials for examination were placed in tetrathionate enrichment broth with brilliant green added. The subsequent isolation and identification procedures were those previously described.[1]

Samples for bacteriological examination were collected from the environment of the processing plant by rubbing sterile cotton-tipped swabs over the surface of the desired areas at various stations along the processing line and from such equipment as tables, pans, knives, saws, et cetera. The swabs were of standard type on applicator sticks and were purchased ready for use. Samples from edible and unedible viscera, rinse waters, and ice baths were taken by inserting the swabs several times into the desired materials in the pans.

FINDINGS

During this five-year period, 155 chickens reacting to pullorum disease were examined. A total of 67 (43.2 %) were positive for *Salmonella pullorum* and 3 (1.9 %) for other Salmonella types. The highest number of *S. pullorum* cultures were obtained from the gall bladder, spleen, and ovary (or oviduct) with 37, 36, and 35 positives, respectively. There were 31 isolations from the heart, 15 from the liver, and 12 from cecal contents. One hen yielded *S. pullorum* from a mesenteric cyst only. *Salmonella cerro* was isolated from the cecal contents of 1 reactor and *Salmonella muenchen* from another. The latter hen yielded *S. pullorum* also from the heart. *Salmonella derby* was isolated from a hard shell egg from the oviduct of a third hen.

Fowl submitted for diagnosis of illness were examined as previously mentioned; in addition, parasitological, virological, and other examinations were performed as indicated. During the same period, 434 birds were examined with 63 (14.5 %) Salmonella isolations, including 24 turkeys with 10 (41 %) isolations of Salmonella. Again, the isolations from the internal organs were predominantly *S. pullorum*, but 3 were infected with *Salmonella typhimurium*, 2 each with *Salmonella miami*, and *Salmonella anatum*, and 1 each with *Sal-*

From the Bureau of Laboratories, Florida State Board of Health, Jacksonville. This work was done under the sponsorship of the Commission on Enteric Infections, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army.

Bacteriologist (Galton) and Veterinarian (Lewis), Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Ga., assigned to Bureau of Laboratories, Florida State Board of Health, Jacksonville.

Mrs. Galton's present address is Communicable Disease Center, Leptospira Research Laboratory, Chamblee, Ga.

Mr. Mackel was previously bacteriologist, Bureau of Laboratories, Florida State Board of Health, now Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Chamblee, Ga.

Veterinarian (Haire), Poultry Service Division, Florida Livestock Board, Jacksonville.

Director (Hardy), Bureau of Laboratories, Florida State Board of Health, Jacksonville.

monella california, Salmonella bredeney, and *Salmonella gallinarum.* More than half of the *S. pullorum* positive birds yielded organisms from the cecal contents also, but of the other Salmonella types, 18 were positive by enteric culture only, 3 by the culture of internal organs only, and 7 by both. Salmonella isolations from internal organs came most frequently from the heart and liver followed by the spleen and gall bladder. The findings are summarized in table 1.

There were 14 different Salmonella types isolated from ill fowl as shown in table 2. *Salmonella pullorum* was encountered most frequently. This type was isolated from 1- to 2-week-old chicks in 12 outbreaks of septicemia with high mortality. In one instance, *S. pullorum* was found in the fluid from an enlarged hock as well as from all internal organs of a 10-day-old chick. One 2-week-old chick yielded *S. pullorum* from the heart and *Salmonella oranienburg* from the cecal contents. Similarly, *S. pullorum* was found in the internal organs of a 10-day-old chick and *Salmonella bareilly* in the cecal contents. *Salmonella anatum* was isolated from encysted egg yolk in the body cavity of a frozen hen carcass. This type was obtained also from the cecal contents of 3 chicks with diarrhea, and from the gall bladder of another bird, 5 months old. *Salmonella anatum* was found in the feces of 1 turkey from which *S. typhimurium* was isolated from other organs and the feces. On four different occasions, *S. bredeney* was isolated from the intestinal contents only of chickens and once from the internal organs only. *Salmonella california* was isolated from the feces of 3 turkeys and also from the heart blood in 1. These came from a large flock with an epidemic of diarrheal disease with high mortality.[3] This type was isolated also from the cecal contents of 2 young chicks. Another outbreak among poults yielded *S. miami* from feces and organs. *Salmonella give* was obtained on two occasions from the cecal contents of apparently healthy 7-day-old chicks from a local hatchery. *Salmonella tennessee* was obtained from 2 chickens submitted for diagnosis of diarrhea and *S. derby* from another. The single isolations of *Salmonella enteritidis* and *Salmonella thompson* were from turkeys, both birds with severe diarrheal disease. The one culture of *S. gallinarum* came from a 3-week-old chick with diarrhea.

According to findings of the authors, birds which come to slaughter are rarely infected. With the cooperation of processing plant owners, birds were removed from the evisceration line immediately after removal of pinfeathers. They were taken to a table, carefully opened, and a portion of the heart, liver, spleen, oviduct, cecum, and muscle obtained for culture. The samples were taken aseptically, the instruments being sterilized by flaming in alcohol after each specimen. None of 872 samples from 129 birds examined in this manner yielded Salmonella. The birds were obtained from the processing lines of the three chicken plants studied over a seven-month period. The number examined during one day was limited to 10.

During the period from August, 1952, through August, 1953, 48 surveys were made at intervals of one to five weeks in processing plants A, B, and C, with findings as shown in tables 3 and 4. Data for 1952 and 1953 were in close agreement, but the percentage of positives varied in the different plants. Of 517 samples from plant

TABLE 1—Source of Salmonella Isolations from Pullorum Reactors and from Sick Fowl, 1949-1953

	Heart	Liver	Spleen	Gall bladder	Ovary or oviduct	Cecal contents	Total positive
Total *pullorum* reactors positive for *S. pullorum*	31	15	36	37	35	12	67*
Total *pullorum* reactors positive for other Salmonella types	0	0	0	0	1**	2	3
Total sick birds positive for *S. pullorum*	32	31	29	28	†	22	35
Total sick birds positive for other Salmonella types	5	5	6	6	1	25	28
Total	68	51	71	71	37	61	133

* 1 culture obtained from mesenteric cyst only; ** from a hard-shell egg in oviduct; † all young chicks or poults.

A, 86 (16.6 %) were positive for Salmonella. In plant B, 97 (19.9 %) of 486 samples were positive, but only 13 (5.3 %) of 241 samples from plant C yielded Salmonella. Salmonella were widely distributed in the environment of the plants but were isolated readily from the viscera and from the areas in which these were handled. The highest percentage of positives was obtained from the edible viscera and from the table on which edible viscera were wrapped, with an average of 31 per cent. It is noteworthy also that the rinse water served as an effective vehicle for the spread of Salmonella. There were 13 (11 %) positives among 118 swabs from tubs holding iced birds ready for market.

Swab samples were taken from the skin surface of the sides of carcasses at various stations along the processing line and cloacal swabs from about half of the birds. In this limited series, Salmonella were isolated more frequently from the sides of the birds, with 8 (2.7 %) of 292 positive, than from cloacal swabs, with 1 positive (1.9 %) in 53 examined.

A local plant processing approximately 1,000 ducks per week provided an opportunity to study the prevalence of Salmonella in the dressed carcasses of these birds. Due to the arrangement in this small plant and to their operating procedures, cultures could not be taken during processing without interfering with the operations. Thus, only a limited number of samples were taken from the environment. Cloacal swabs taken immediately after defeathering yielded Salmonella in 126 (24.8 %) of 507 birds. Of 20 samples taken from the defeathering machine, 16 (80.0 %) were positive, while only 4 (7.5 %) of 53 samples taken from materials during evisceration yielded Salmonella. The method of processing in this duck plant differed considerably from procedures in other poultry plants studied. Immediately after defeathering, the bird is dipped into a hot wax bath, then into cold water. After removal of the wax by hand, the bird is placed in a cold room for several hours before evisceration.

During these studies 213 Salmonella isolations were obtained from the environment of chicken processing plants and from processed birds and 147 from ducks and materials in this processing plant as shown in table 2. There were 21 different serotypes including 1 new type.[4] Although *S. typhimurium*, a type frequently incriminated in outbreaks of human infection, was most prevalent, it did not occur with regularity. For example, 21 of the 36 isolations in plant A were obtained in three surveys one week apart and the remaining 15 cultures were isolated during two sur-

TABLE 2—Distribution of Salmonella Isolations by Type in Poultry and Poultry Processing Plants in Florida, 1949-1953

Type	Diagnostic exam.			Chicken processing plants				Ducks and duck processing plant
	Pullorum reactors	Ill birds	Total	A	B	C	Total	
S. typhimurium		3	3	36	40	7	83	62
anatum		7	7	13	10	1	24	80
pullorum	67	36	103		1		1	
16:z29				24*			24	
tennessee		2	2	1	13	2	16	
give		2	2	1	6		7	4
newington					11		11	
infantis					9		9	
montevideo				4	2		6	
gallinarum		1	1	2	3		5	
bredeney		5	5		1		1	
newport					5		5	
orion				5			5	
california		5	5					
bareilly		1	1	3	1		4	
muenchen	1		1			2	2	1
derby	1	1	2		2		2	
oranienburg		1	1			2	2	
kentucky				2			2	
thompson		1	1		1		1	
miami		2	2					
sandiego					2		2	
cerro	1		1		1		1	
enteritidis		1	1					
TOTAL	70	68	138	91	108	14	213	147

* Isolated during 3 different surveys, 6/29/53, 7/13/53, 8/24/53.

veys three and four weeks later. This type was not encountered in 13 other surveys in plant A. Similarly, 28 of the 40 *S. typhimurium* isolations from plant B occurred in two surveys one week apart. The remaining 12 cultures appeared widely distributed in five of 16 other surveys. The 24 cultures of Salmonella type, 16:z29 were isolated during three different surveys, on June 29, July 13, and August 24, 1953, with 17, 6, and 1 isolations, respectively, being made. Birds processed during the first of these surveys had been received from the area around Gainesville, Georgia.

The five cultures of *Salmonella orion* were encountered during a single survey in plant A. In plant B the predominance of one type during a single survey was further emphasized by the occurrence of all 13 cultures of *S. tennessee* from one survey, all 11 of *Salmonella newington* from another survey, and the 9 of *Salmonella infantis* from a third survey taken over an eight-month period. Although the plant was visited at frequent intervals, these three types were not encountered again. *Salmonella gallinarum* was found twice in plant A while hens were being processed. During this same survey, 13 Arizona paracolon cultures type 10:1, 2, 5 were isolated. The three cultures of *S. gallinarum* from plant B were found only once, shortly after hens had been processed. The isolation of *S. pullorum* only once may be attributed to the effectiveness of the Pullorum Disease Control Program of the Florida Livestock Board. All Salmonella types encountered in fowl and poultry processing plants with the exception of *S. gallinarum* and the new serotype 16:z29 have been found in human beings in Florida.

TABLE 3—Isolation of Salmonella by Swab Testing of Various Materials in Poultry Processing Plants

Source of specimen	Cultured	Positive for Salmonella (No.)	(%)
Bleeding trough	63	0	
Table on which oil glands are removed	84	14	16.7
Rinse water on above table	30	5	16.7
Table on which edible viscera are removed	125	15	12.0
Rinse water on above table	69	4	5.8
Table on which unedible viscera are removed	120	24	20.0
Rinse water on above table	73	8	10.9
Gizzard peeler	48	2	4.2
Water conveyor for gizzards and liver	20	1	5.0
Tray with edible viscera	140	43	30.7
Saw to remove feet and necks	143	29	20.3
Rinse water for saw	40	8	20.0
Table on which edible viscera are wrapped	39	12	30.8
Table at which edible viscera are replaced in bird	59	9	15.2
Rinse water on above table	2	0	
Table at which birds are graded	24	3	12.5
Iced tubs with marketable birds	118	13	11.0
Miscellaneous	47	6	12.8
TOTAL	1,244	196	15.8

TABLE 4—Prevalence of Salmonella in Poultry and Poultry Processing Plants in Florida, 1949-1954

Source of culture	Specimens examined	Positive (No.)	(%)
Diagnostic examinations of all birds	434	63	14.5
Positive reactors to pullorum whole blood test	155	69	45.8
Tissues from 129 presumably healthy broilers	872	0	0.0
Materials in processing rooms (chickens)	1,244	196	15.7
Sides of chickens during processing	292	8	2.7
Cloacal swabs on slaughtered chickens	53	1	1.9
Cloacal swabs on slaughtered ducks	507	126	24.8
Materials in processing room of duck plant	53	4	7.5
Defeathering machine in duck plant	20	16	80.0
Total cultures	3,630	483	13.3

DISCUSSION

The method of processing appeared to have some influence on percentage of positives. For example, in plant C which yielded the lowest percentage of positive samples, the evisceration table was equipped with running rinse water. The edible viscera were separated from unedible viscera, rinsed under this running tap and passed directly to the wrapping table. This plant yielded only 1 of the 51 positive samples from trays or tables containing edible viscera. In contrast, rinse water for edible viscera in plants A and B was held in pans, this water being changed only when the pans were filled.

The preferred procedures for dressing and evisceration of poultry have been adequately reviewed by Kyle, McFadden, and Gunderson.[2] In the three local chicken processing plants and one duck processing plant studied, the physical facilities and sanitary conditions varied considerably and the omission of certain steps, such as singeing and washing as outlined by Kyle

et al., was notable. Of those processing chickens, plant A was large and well equipped. It was of galvanized metal construction with concrete floors. The walls and ceilings appeared clean. The batteries of chickens were kept in a large room adjoining the dressing and evisceration room. Doors into the latter room were screened. However, little attention was given to the refuse (feathers and unedible viscera) which collected on the floors of this room. This plant was equipped to process 960 birds per hour; however, their average output ranged between 10,000 and 12,000 per week. It was the only plant equipped with a spray-type washer to cleanse the outside of the bird before final icing.

Plant B was moderately well equipped but had only about one half the space of plant A. It was of concrete block construction with concrete floors and handled between 20,000 and 25,000 birds per week. Walls and ceilings appeared to receive little attention and floors along the evisceration line were usually covered with refuse. There were no screens, and flies were abundant in the entire plant. The batteries of chickens were kept in a room adjoining the dressing and eviscerating room. The toilet room opened directly into the dressing and evisceration room less than 3 feet from the point where ice baths containing marketable birds were stationed.

Plant C was considerably smaller than plant B and of the same concrete block construction although more than two thirds of the floor space of the building was used for batteries of live birds and the cold room for dressed birds. The killing, dressing, eviscerating, and grading procedures were all carried out in the remaining one-third area of this room. The plant processed approximately 8,000 to 10,000 birds per week.

The fact that no Salmonella were isolated during 13 of the 48 surveys in these three plants is not surprising in view of the failure to find Salmonella in cultures from 129 broilers being processed and the low percentage of Salmonella obtained from cloacal swabs on 53 birds during processing (1, or 1.9 %). The evidence indicates, however, that infected birds do come to slaughter and that Salmonella are widely disseminated in the environment of the plant during processing. Swab samples taken from materials in the environment of these plants revealed 15.8 per cent Salmonella from 1,244 samples. There were 13 (11 %) positives from 118 swabs from tubs holding iced birds ready for market. The organisms on these contaminated birds, preserved by refrigeration, would reach the kitchens and the hands of cooks and thus provide a ready source of salmonellosis in man. There is no clear evidence whether Salmonella persist or multiply in a contaminated plant or whether they are merely disseminated from infected to uninfected birds during concurrent processing. The isolation of specific types in substantial numbers on one day only suggests the latter. For example, in plant B all of the 13 cultures of *S. tennessee* were obtained during one survey; similarly, all 11 *S. newington* cultures were isolated at one time, and the 9 cultures of *S. infantis* at another. In spite of repeated visits at weekly intervals to this plant, these types were not found again.

These bacteriological studies are too limited to provide a measure of the sanitary significance of the differences in processing techniques. They do indicate, however, the need for establishing bacteriological standards for adequate detection of these pathogenic organisms so that acceptable products reach the consumer.

SUMMARY

Bacteriological examination of 155 chickens reacting to pullorum disease yielded 67 (43.2 %) cultures positive for *S. pullorum* and 3 (1.9 %) for other Salmonella types. During the same period 434 chickens and turkeys submitted for diagnosis of illness yielded 63 (14.5 %) Salmonella, of which 35 were *S. pullorum*.

Data on the prevalence of Salmonella in three poultry processing plants have been presented. In all, 1,244 cultures were taken from materials in the plants, and 196 (16 %) were positive for Salmonella. The highest percentage of positive findings was from the edible viscera and the table on which edible viscera were wrapped. In addition, 507 cloacal swabs from ducks in another processing plant yielded Salmonella from 126 (24.8 %). A total of 24 Salmonella serotypes including one new type were isolated from fowl and from the environment of poultry processing plants.

These bacteriological studies emphasize the urgent need of detailed attention toward bacteriological standards and all other phases of the sanitary control of poultry processing plants.

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The Use of Aqueous Humor for Early Diagnosis of Newcastle Disease

D. S. CLARK, D.V.M.; E. E. JONES, D.V.M.; F. K. ROSS

San Gabriel, California

LIVESTOCK and poultry diagnostic laboratories are concerned with methods of diagnosis which are practical, rapid, and accurate during the early stage of a disease. Various serological and biological tests are available for diagnosis of Newcastle disease, and the literature contains much information regarding these tests. Virus isolation, which is the most positive diagnosis, requires several days for completion. It is of great value for research purposes but is not always practical in a busy diagnostic laboratory because of the time required for its completion.

The serum neutralization (SN) test is not reliable until the later stages of Newcastle disease, when the antibodies have developed in the serum of infected birds. In addition, egg inoculation work requires several more days to complete.

The hemagglutination-inhibition (HI) test, until recently, has been the most practical and rapid diagnostic aid. However, this test also is not applicable in the early stages of an outbreak, when HI bodies have not yet developed in the blood stream. Also, in some subclinical but harmful outbreaks, antibodies apparently fail to develop in sufficient quantities to produce a positive HI reaction.

In previously vaccinated flocks, both the HI and SN tests can not be correctly evaluated since a positive reaction may result from use of live virus vaccine.

The presence of Newcastle disease virus can be demonstrated by means of an hemagglutination (HA) test. However, until recently no practical test utilizing this phenomenon as a diagnostic aid during the first seven days of infection has been developed.

A procedure for rapid diagnosis, using lung extract, was described by McClurkin, Sinha, and Hanson, in 1954.[1] By this technique, HA activity is demonstrated in fluids extracted from lungs which have been frozen. The lung extract is adjusted to pH 7 and treated for an hour in a water bath before use. Newcastle disease antiserum, used in the extract HA test, is recommended to determine the specificity of the hemagglutination.

Recently, a group of chicks 5 to 11 weeks of age was submitted to the San Gabriel laboratory for diagnosis. The history indicated a serious condition, characterized by an inflammation of the eyes, lacrimation, and blindness. The disease had spread to birds of various ages, and treatment was said to be of no avail. The lesions were confined to the eyes, in which there was a conjunctivitis and blepharitis, resulting in profuse lacrimation. The corneas of some individuals were opaque, and a few had corneal ulcers. The common respiratory infections and fowl pox were eliminated by laboratory tests. Bacteriological cultures were negative.

Aqueous humor was drawn from the eyes of several birds and pooled. The lungs of the same birds were removed and frozen, after which the fluid was extracted and pooled. Parallel HA tests were made with the two fluids. The HA titer of the aqueous humor was 1:80 and that of the lung extract was 1:40. Newcastle disease virus was isolated from the aqueous humor by chicken embryo inoculation. The HI test of the serum was negative at that time, but later in the outbreak there was a positive reaction.

To ascertain if the lung extract and aqueous humor of healthy, unexposed chicks would produce hemagglutination, 10 chicks 1 week of age, and 10 chicks 4 weeks of age were tested and found to be negative.

A laboratory study was initiated to further explore the comparative values of the lung extract and the aqueous humor for an HA test. As a further comparison, the HI test was included.

METHODS AND RESULTS

White Leghorn cockerels 6 weeks of age, brooded in the laboratory free from exposure to disease, were used. Thirty-five chicks were isolated, wing banded, and inoculated intramuscularly with a dosage of Newcastle disease virus known to be lethal. Four days after inoculation, 12 chicks exhibited signs of Newcastle disease. They were bled for the HI test and destroyed. The lungs were removed and processed according to the technique described by McClurkin *et al.*[1] The aqueous humor was removed by aspirating with a syringe and a 19-gauge needle. Each bird yielded approximately 1 cc. of nearly clear fluid. The fluid samples were divided, one portion being

From the California State Department of Agriculture, Livestock and Poultry Pathology Laboratory, San Gabriel, Calif.

used in the fresh state and the remainder frozen. No significant difference was observed in the hemagglutination activity of the fresh and the frozen portions. The aqueous humor was not heat treated and the pH was not adjusted. The technique used for the HA test of the lung and aqueous humor was the same as described by Beach[2] in 1948.

The remainder of the chicks were destroyed as symptoms developed. Tissues were removed and tested in the same manner.

It will be seen in table 1 that the HA reactions occurred early and continued for approximately ten days. There were variations in titers between the lung extract and aqueous humor but, in general, the results compared favorably. Hemagglutination inhibition was not regularly demonstrated until the seventh day, or later.

Subsequently, 20 field cases of suspected Newcastle disease were selected for further studies. The results of the HA tests, using lung extract and aqueous humor, along with the HI test are recorded in table 2.

The final diagnosis of these field cases correlated favorably with the HA and HI test results. In three instances, the lung extract gave better results than the aqueous humor, and the reverse was true in 5 other cases. In instances of complete negative reactions, usually some other disease was involved. In groups 5, 11, and 16, both fluids produced hemagglutination in a low titer and there was a low HI titer in No. 11. There was no evidence of Newcastle disease in these chicks. The significance of low titer reactions needs further investigation. When they occur, a guarded diagnosis is suggested. Differential diagnosis, with the use of Newcastle disease and fowl plague antiserums in questionable cases, was not fully explored.

TABLE 1—Results of the Laboratory Trial of the Hemagglutination (HA) Test, Using Lung Extract and Aqueous Humor, Along with the Hemagglutination-Inhibition (HI) Test

Days after inoculation	Test bird (No.)	HI	HA	
			Lung extract	Aqueous humor
4	1	Neg.	Neg.	1:20
4	2	Neg.	1:40	1:20
4	3	Neg.	1:80	1:80
4	4	Neg.	1:80	1:80
4	5	Neg.	1:40	1:40
4	6	Neg.	1:40	1:40
4	7	Neg.	1:40	1:40
4	8	Neg.	1:20	1:80
4	9	Neg.	1:20	1:40
4	10	Neg.	1:40	Neg.
4	11	Neg.	Neg.	1:80
4	12	Neg.	1:160	1:80
4	13	Neg.	1:10	1:40
5	14	Neg.	1:10	1:10
5	15	Neg.	Neg.	Neg.
5	16	Neg.	1:20	1:40
5	17	Neg.	Neg.	1:40
6	18	1:20	Neg.	1:20
6	19	Neg.	1:40	1:80
6	20	1:40	1:40	1:10
6	21	Neg.	1:20	1:40
6	22	1:10	1:10	1:10
7	23	Neg.	1:40	1:80
7	24	Neg.	1:80	1:80
7	25	1:80	1:20	1:40
7	26	1:320	1:40	1:40
7	27	1:20	1:20	1:20
8	28	1:160	1:40	1:20
8	29	1:40	Neg.	1:80
8	30	1:40	1:40	1:20
10	31	1:320	1:40	Neg.
10	32	1:160	1:40	1:40
14	33	1:320	1:20	Neg.
14	34	1:160	Neg.	Neg.
14	35	1:160	Neg.	Neg.

TABLE 2—Results of the Field Trial of the Hemagglutination (HA) Test, Using Lung Extract and Aqueous Humor, Along with the Hemagglutination-Inhibition (HI) Test

Case (No.)	Days after symptoms observed	HA		HI	Final diagnosis
		Lung extract	Aqueous humor		
1	7	Neg.	1:80	1:80	Newcastle disease
2	3	Neg.	Neg.	Neg.	Infectious coryza
3	?	1:80	1:40	Neg.	Newcastle disease
4	0	Neg.	1:80	Neg.	Newcastle disease
5	3	1:10	1:10	Neg.	No diagnosis
6	7	Neg.	Neg.	Neg.	Laryngotracheitis
7	7	Neg.	1:640	Neg.	Newcastle disease
8	7	Neg.	1:640	No test	Newcastle disease
9	10	1:160	1:160	1:160	Newcastle disease
10	10	1:40	1:160	1:320	Newcastle disease
11	13	1:20	1:20	1:20	Infectious coryza
12	7	Neg.	Neg.	Neg.	Air sac infection
13	7	Neg.	Neg.	Neg.	Pox vacc. reaction
14	1	Neg.	Neg.	Neg.	Infectious bronchitis
15	7	Neg.	Neg.	Neg.	Laryngotracheitis
16	1	1:20	1:20	Neg.	Hemorrhagic disease
17	14	1:20	Neg.	1:40	Newcastle disease
18	7	1:40	1:40	Neg.	Newcastle disease
19	10	1:40	1:10	1:40	Newcastle disease
20	10	1:80	1:80	1:20	Newcastle disease

SUMMARY

Newcastle disease virus was demonstrated in the aqueous humor of naturally and artificially infected chicks. Untreated or previously frozen aqueous humor of infected chicks agglutinated chicken red blood cells, while the uninfected material did not. The limited study indicates that aqueous humor, as a source of virus for the hemagglutination (HA) test, compares favorably with extract of frozen lung tissues for an early and rapid presumptive diag-

nosis of Newcastle disease in chickens. The hemagglutination-inhibition (HI) test is helpful seven days or more after exposure. A combination of the HA and HI tests is suggested.

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Local Inhibition by Estrone of Testosterone-Induced Growth of the Capon's Comb

JOHN E. MARTIN, V.M.D.; JOHN H. GRAVES, D.V.M.; F. C. DOHAN, M.D.

Philadelphia, Pennsylvania

THE SITE (or sites) of action of the antagonistic effect of estrogens upon the growth of the capon's comb induced by androgens is not definitely known.

Boas and Ludwig [1] have reviewed the literature. They concluded from their experiments that inhibition of comb growth in cockerels by locally applied estrogens is dependent upon suppression of gonadotropin secretion in the adenohypophysis, thereby depressing androgen output by the gonads. In contrast, Emmens and Bradshaw [2] and Mühlbock [3-5] have demonstrated that estrogens are considerably more potent by local application than by intramuscular injection in their capacity to antagonize comb growth induced in the capon by exogenous androgens. These results in the capon indicate that the antagonism may occur by other than indirect inhibition of gonadal function and offer support for the view that an antagonistic effect of estrogens may occur at the comb tissue level. However, variations in the rates of absorption and other unknown factors may account for the difference in effectiveness of injected and locally applied estrogen, and the possibility of a central antagonistic effect is not completely eliminated. Since the evidence that the antagonism may occur at the target tissue level seemed inconclusive, we investigated the problem using the capon's comb which had been surgically separated into fore and hind portions. This procedure produced a paired organ and thus afforded an opportunity to study the effect of a locally applied antagonistic agent on one organ, using the other as a control. Testosterone propionate was applied to both half-combs and estrone to one.* An antagonistic effect at the target tissue level has been demonstrated in this fashion.

MATERIALS AND METHODS

One-day-old White Leghorn male chicks were obtained from a local hatchery and raised in a battery-type brooder for four weeks. During this period, they were fed a standard starting mash. The birds were then moved to larger cages and placed on an "all purpose" growing mash. When 4 to 6 weeks old, they were caponized. After recovery from this operation, a wedge-shaped portion of about 5 mm. wide was removed from the center of the comb, so that two entirely separate "half-combs" were produced. These were designated "fore comb" and "hind comb." A period of four weeks was allowed after caponization before any experiments were started. Completeness of caponization was established by measuring the combs several times after the operation to determine that they were not growing. On some birds, this was further verified by removing a small portion of the combs for histological study. In most instances, the birds were 8 to 10 weeks old when the experiments were started, but in some cases birds were re-used so that they were older. If birds were used more than once, a waiting period was allowed between experiments for the combs to return to their original size.

Solutions of testosterone propionate and estrone, and mixtures of the two hormones, were prepared in sesame oil at the beginning of each experiment. The solutions were made so that the individual daily dose of each hormone, or of the hormone mixture, was contained in 0.05 cc. of oil.

On the day when hormone applications were started, the length and the height of each half-comb were measured in millimeters with calipers, and the sum of these two measurements was taken as an index of the initial comb size. Hormone solutions were then applied to each half-comb once daily for five days. Applications were made from a syringe calibrated in 0.01-cc. graduations and equipped with a 22-gauge needle. Solutions were dropped on the comb so that the entire surface was covered with oil without the necessity of rubbing the comb with the needle. Twenty-four hours after the last hormone application, the length and height of each half-comb were again measured, and the sum was compared with the control measurement to determine the change in comb size. Growth of half-combs on each bird was obtained and the data subjected to an "individual comparison," using the formula * of Snedecor.[6] "P" was determined from the tables of Fisher.[7] The term "significant" as used in the text indicates that there was less than 1 chance in 20 that the difference was due to chance variation (*i.e.*, $P < 0.05$).

From the Department of Physiology and Pharmacology and Department of Anatomy, School of Veterinary Medicine, and the Department of Medicine, School of Medicine, University of Pennsylvania, Philadelphia.

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* Testosterone propionate and estrone were supplied through the courtesy of Mr. Fred Houghton, Ciba Pharmaceutical Products, Inc., Summit, N. J.

* $t = \frac{\bar{x}}{S\bar{x}}$ where $\bar{x}$ = the difference in the means and $S\bar{x}$ = the standard error of the differences in the means.

On some birds, sections of the fore and hind combs were taken for microscopic study at the beginning and end of the treatment period. After fixation, the tissues were sectioned at 6 μ thickness and stained with hematoxylin and eosin.

RESULTS

Results are shown in table 1 and figures 1 to 5. Eleven separate experiments were performed, using from 5 to 20 birds each. The birds were divided into five groups according to dosage of estrone. Group 1 received 2 μg. of testosterone propionate daily on both half-combs. Groups 2, 3, 4, and 5 were treated with similar daily doses of testosterone propionate on both half-combs but, in addition, received 80, 100, 160, and 200 μg., respectively, of estrone on one half-comb (table 1). In each group about one half were treated with estrone on the fore combs and the others on the hind combs. Group 1 was designated as the treated control group but, in using topical applications with the double-comb technique, each bird acted as its own control since two separate halves of the tissue were under study on each bird. Thus, in each bird, growth of the half-comb treated with testosterone propionate plus estrone was compared to growth of the adjacent half-comb receiving only testosterone propionate. Any significant depression of growth in the estrone-treated half-comb was considered to be the result of a local antagonistic effect of estrone on the growth-producing action of testosterone propionate. Similarly, any marked difference in the histological structure of the half-combs on an individual bird was taken as further evidence of a local effect of estrone.

Comb Growth.—In the 23 birds receiving only testosterone propionate on both half-combs (group 1), all combs grew.

TABLE 1—Average Growth of Half-Combs After Five Days of Treatment

Group	No. birds	Daily dose (μg.) TP* both combs	Daily dose (μg.) Estrone one comb	Comb growth (mm.) TP only	Comb growth (mm.) Estrone comb	S.E.†
				5.10		
1	23	2	0	4.55	.....	0.39
2	15	2	80	3.59	2.79	0.50
3	30	2	100	3.78	1.55	0.38
4	25	2	160	3.80	3.02	0.54
5	15	2	200	3.23	0.50	0.69

* TP—Testosterone propionate; † S.E.—standard error of the difference of the means.

There was some difference in the growth of the fore and hind combs on individual birds (fig. 1) and the average growth of fore combs was somewhat greater than that of hind combs (Table 1). However, these differences were not statistically significant and the results indicate that either the fore or hind comb is capable of responding similarly to testosterone propionate.

Both the 100- and 200-μg. doses of estrone (groups 3 and 5) produced a highly significant inhibition of growth in the half-comb to which they were applied ($P < 0.001$ and 0.01, respectively). Average growths for the groups indicate that the half-combs treated with 100 μg. of estrone grew only 41 per cent of their adjacent controls, while those treated with 200 μg. of estrone grew only 15 per cent as much as their adjacent controls (table 1). Individual birds of the two groups showed some variation, but in nine of 30 half-combs treated with 100 μg. of estrone and in six of 15 treated with 200 μg., there was either complete failure of growth or even a slight decrease in size (fig. 1).

Neither 80 or 160 μg. of estrone (groups 2 and 4) produced a statistically significant inhibition of growth in half-combs to which it was applied. However, each group does show 3 birds in which the estrone-treated half-comb failed to grow (fig. 1).

Growth (table 1) of half-combs receiving only testosterone propionate in groups 2, 3, 4, and 5 is less in every case than that in group 1. It appears likely that this depression of growth is a result of some estrone being absorbed from the half-comb to which it was applied and being carried in the general circulation to the opposite half-comb where it exerted a slight inhibitory effect.

Histological Examination.—Results of our histological examination on capons and cockerels generally confirm those already reported,[8, 9] so that only a brief description of the structure need be given.

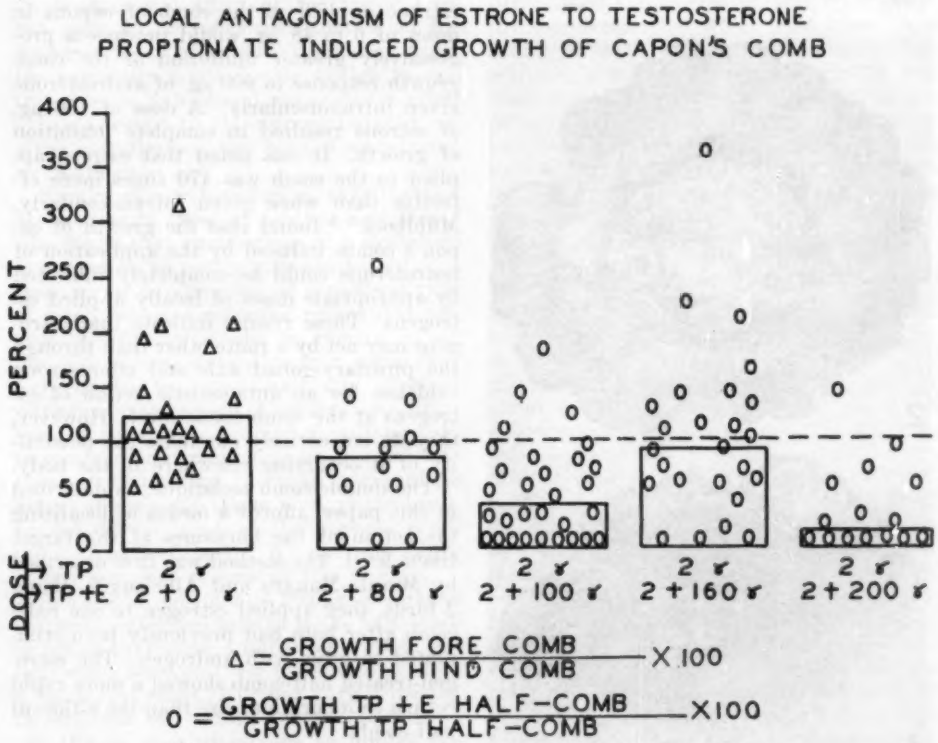
The cockerel's comb (fig. 2) consists of an epidermis and dermis. The latter may be divided into three well-defined layers: (*a*) the central layer, which is a core of rather dense connective tissue containing blood vessels, nerves, and some adipose tissue; (*b*) the intermediate layer made up of loose collagenous fibers widely sep-

arated by an abundance of intercellular ground substances; and (*c*) a narrow subepithelial layer of rather dense connective tissue containing a plexus of blood vessels.

After castration, the most significant change occurs in the intermediate layer. This decreases in size and the collagenous fibers become closely packed owing to an apparent loss of intercellular ground substance. There is no longer a sharp demarcation between the various layers of the dermis (fig. 3).

In a few cases, histological examinations were made of the combs of capons in which both half-combs were treated with testosterone propionate alone. The combs showed a return toward a differentiated type of structure characteristic of the cockerel comb. Again, the most striking change occurred in the intermediate layer which became sharply demarcated from the other layers. There was an increase in intercellular ground substances separating the collagen fibers and the relative size of the layer increased.

The histological structure of half-combs of 12 birds from group 5 was studied before and after treatment. Before treatment the combs had the typical capon-type of structure. After treatment, the half-comb to which only testosterone propionate was applied had the histological appearance of a cockerel comb or approached it in varying degrees (fig. 4). In general, the greater the comb growth, the larger the relative size of the intermediate zone. The adja-



TP—testosterone propionate; TP + E—testosterone propionate plus estrone.

Fig. 1—The percentage of growth of half-combs treated with a mixture of testosterone propionate plus estrone compared to growth of adjacent half-comb treated with testosterone propionate only. Birds depicted in first column at the left received only testosterone propionate on both half-combs. Solid lines at top of columns indicate average for each group.

cent half-comb treated with testosterone propionate plus estrone retained the undifferentiated type of capon structure or changed only slightly (fig. 5).

DISCUSSION

An antagonistic effect of one steroid upon the growth-stimulating action of another on a particular target tissue has been demonstrated for several types of compounds and tissues and has been postulated for others. The exact mechanism of this effect is not known but, among the numerous possibilities, the most probable explanations appear to be: (*a*) inhibition of the production of the stimulating hormone and (*b*) an antagonistic effect within the target tissue. Experimental evidence to support either of these concepts has been supplied by workers using the chicken's comb as the test tissue.

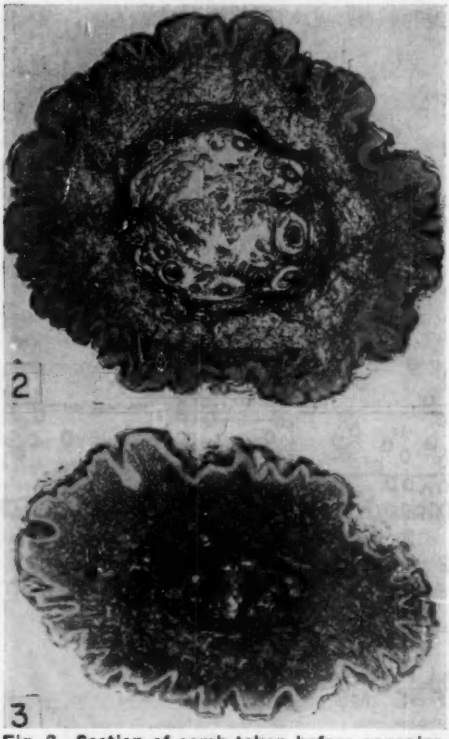


Fig. 2—Section of comb taken before caponization showing differentiated structure of cockerel comb.

Fig. 3—Section of comb taken four weeks after caponization demonstrating undifferentiated capon structure.

Boas and Ludwig [1] reported that estradiol applied locally markedly inhibited growth and development of the comb in young cockerels but had no apparent effect on the comb response (growth or histological development) to exogenous testosterone. They concluded that estrogens inhibit comb growth by suppressing gonadotropin secretion by the adenohypophysis, thereby reducing androgen output by the gonads. They used large doses of testosterone (50 μg.) and relatively small amounts of estradiol (5 to 100 μg.) which may partially account for their failure to observe any significant effect of estradiol on the action of exogenous testosterone.

Emmens and Bradshaw [2] showed that estrone applied to the comb of capons in doses of 6 to 48 μg. would produce a progressively greater inhibition of the comb growth response to 600 μg. of androsterone given intramuscularly. A dose of 150 μg. of estrone resulted in complete inhibition of growth. It was noted that estrone applied to the comb was 470 times more effective than when given intramuscularly. Mühlbock [3-5] found that the growth of capon's combs induced by the application of testosterone could be completely inhibited by appropriate doses of locally applied estrogens. These results indicate that estrogens may act by a route other than through the pituitary-gonad axis and offer strong evidence for an antagonistic action of estrogens at the comb tissue level. However, they do not entirely eliminate the possibility of it occurring elsewhere in the body.

The double-comb technique, as described in this paper, affords a means of localizing the action of the hormones at the target tissue level. The method was first described by Morato-Manaro and Albrieux.[10] Using 2 birds, they applied estrogen to one half-comb after both had previously been stimulated to grow with androgen. The estrogen-treated half-comb showed a more rapid return to its original size than the adjacent half-comb.

In experiments reported here, the topical application of 100 or 200 μg. of estrone to a half-comb also receiving 2 μg. of testosterone propionate resulted in a highly significant inhibition of growth and development when compared to the adja-

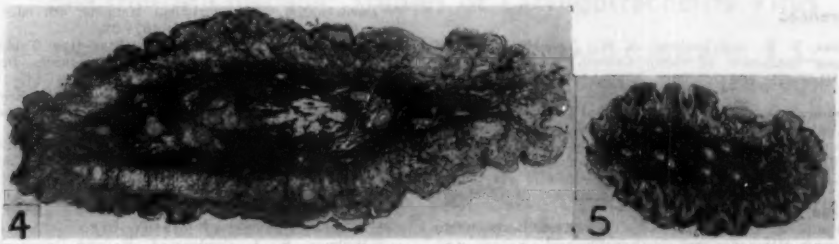


Fig. 4—Section of half-comb after application of 2 μg. of testosterone propionate daily for five days. Note differentiated structure, similar to that of cockerel.

Fig. 5—Section of adjacent half-comb after application of 2 μg. of testosterone propionate plus 200 μg. of estrone daily for five days. Note that this half-comb retained the undifferentiated structure characteristic of capon.

cent half-comb treated only with testosterone propionate. These results are compatible with the view that estrone was exerting its antagonistic effect at the tissue level but they do not offer an explanation for its exact mode of action. Similarly, using the surgically made double vagina in castrated mice, a localized antagonistic effect of testosterone to the estrone-induced vaginal cornification has been demonstrated by Mellman *et al.*[11]

Results of our histological examinations are similar to those of Ludwig and Boas[9] who found that applications of testosterone to the combs of young male chicks caused a more rapid development of a cockerel type of comb, whereas estradiol retarded development, causing combs to remain in an undifferentiated state. However, while they were unable to demonstrate any effect of estradiol on the action of testosterone, when both of these were applied simultaneously to combs of cockerels, our studies indicate that estrogens may exert an antagonistic action at the comb tissue level on the histological development of the capon's comb induced by androgen. We are not aware of any previous reports of this finding.

Since both the 100 and 200 μg. of estrone markedly inhibited growth in the half-comb to which they were applied, one would expect doses between these two levels to exert a significant effect. Further work is needed to establish the reason for the failure of the 160-μg. dose of estrone to inhibit the effect of testosterone propionate. A possible explanation might be based on the relative importance of the ratio of stimulating to inhibiting hormone in determining the activity of a steroid pair. Grauer, Starkey, and Saier[12] have reported a somewhat related finding in which the proportion, rather than total amounts, of two steroids determined the degree of activity. Dehydroisoandrosterone, when used alone, was found to be much less active than androsterone in stimulating comb growth, but when combined with a small amount of the latter substance, its activity was greatly increased. However, when the reverse situation prevailed (*i.e.*, a small amount of dehydroisoandrosterone combined with a large amount of androsterone) the activity of the androsterone was decreased.

Summary

Combs of White Leghorn capons were surgically separated into two half-combs. A daily dose of 2 μg. of testosterone propionate was applied to both half-combs and simultaneous applications of various doses of estrone were made to one. The steroids were applied for five days. Half-combs treated with estrone in daily doses of 100 or 200 μg., but not with 80 or 160 μg., grew significantly less than their paired controls receiving only testosterone propionate. Histological development of the cockerel-type comb occurred in half-combs receiving only testosterone propionate, but the development was markedly depressed in the corresponding half-comb treated with the estrone-testosterone propionate mixture. It is concluded that, under conditions of this experiment, estrone acted by antagonizing the action of testosterone propionate at the target tissue level. Relative proportions of hormones present may be an important feature in determining the type of response to the mixture.

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Electron Microscopic Studies of Laryngotracheitis Virus

REGINALD L. REAGAN, Major (Ret.) U.S.A.; EDWARD C. DELAHA, B. S.; SUE R. COOK, B.A.; ARTHUR L. BRUECKNER, V.M.D.; JAMES E. PORTER, D.V.M.; IRVIN M. MOULTHROP, D.V.M.

College Park, Maryland

IN 1930, Beach [1] showed avian infectious laryngotracheitis to be a specific virus disease.

He found that it could be filtered readily and Gibbs [2] estimated the virus particle to be between 45 and 85 mμ. The virus is found only in the air passages and is infective only by introduction in that way, except that, rarely, infection can be established by intravenous inoculation. Egg-propagated virus, when dried and kept in a refrigerator, retained its poteney and immunizing properties for 421 days.[3] Brandly [4] in 1936 cultivated the virus on the chorioallantoic membrane of developing chicken embryos. The virus produces white plaques on this membrane.[5]

The laryngotracheitis virus used in this study was obtained from Dr. F. R. Beaudette at Rutgers University. One milliliter of lyophilized virus-infected chorioallantoic membrane (strain L5228, 188 passage) was received at this laboratory and made into a 10 per cent suspension with physiological saline. Fifteen 9-day-old embryonating eggs were inoculated aseptically on the chorioallantoic membrane with this suspension. Five more embryonating eggs were inoculated on the chorioallantoic membrane with sterile physiological saline. Both sets of eggs were incubated at 37 C. and were candled twice daily. On the fifth day postinoculation, the membranes of the virus-injected eggs were congested and thickened. The embryos were destroyed, and the membranes were aseptically recovered from the infected eggs. Membranes of the control eggs remained normal but were also removed five days postinoculation. The pools of infected chorioallantoic membranes and the pools of normal chorioallantoic membranes were ground separately with alundum and diluted to a 20 per cent suspension with physiological saline. The two suspensions were then subjected to five minutes of centrifugation in an angle centrifuge at 2,000 r.p.m. The supernatants were recovered from the specimens and filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation for two hours at 42,000 r.p.m. in the Spinco ultracentrifuge under refrigeration. The temperature of the refrigerated outer jacket stayed constant at —8 C. during centrifugation. After the two-hour centrifugation period, the supernatants from the two specimens were discarded, and the sediment from each was resuspended in 1 ml. of distilled H_2O. Several drops of each suspension were placed on several parlodion film supports which had been prepared seventy-two hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium [6] at arc tangent 4/14 and examined under the R.C.A. electron microscope, type E.M.U. The balance of the infected specimen was inoculated into the air passages of 2 immunized chickens and 2 nonimmunized chickens. Within five days the 2 susceptible chickens showed symptoms of laryngotracheitis. The 2 chickens immunized against laryngotracheitis failed to show any symptoms of the disease.

RESULTS

Upon electron microscopic examination of the concentrated suspension of the chorioallantoic membranes inoculated with

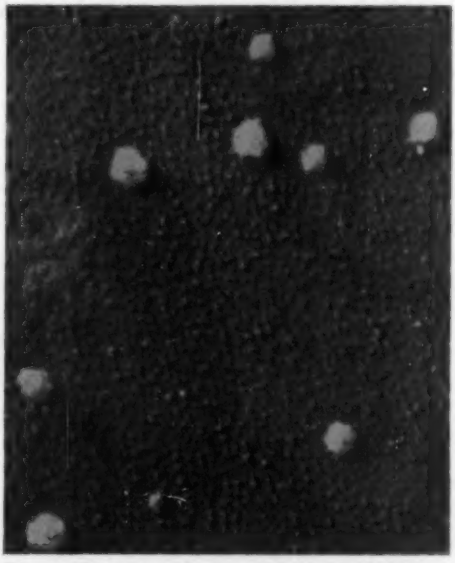

Fig. 1—Electron micrograph of laryngotracheitis virus. Shadowed with chromium at tangent 4/14. x 40,000.

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park; professor of virus diseases (Reagan), virologist (Delaha and Cook), professor and director of Live Stock Sanitary Service (Brueckner), associate professor (Porter), professor (Moulthrop).

normal physiological saline, no virus-like particles could be seen. Upon examination of the concentrated specimen from the chorioallantoic membranes infected with laryngotracheitis, uniform virus-like particles were demonstrated as shown by figure 1. These bodies measured 100-110 mμ by direct measurement and were spherical in shape.

SUMMARY

Studies by electron microscopy of chorioallantoic membranes of chicken embryos infected with laryngotracheitis virus show the virus to be spherical in shape with a diameter of 100-110 mμ. These bodies could not be demonstrated in concentrated suspensions of normal chorioallantoic membranes subjected to the same procedure of concentration and examination. When a portion of the concentrated infected chorioallantoic membrane suspension was injected into the air passage of 2 immunized chickens and into 2 susceptible chickens, the 2 nonimmunized ones developed symptoms of laryngotracheitis while the 2 immunized ones remained normal. This indicated the virus to be that of laryngotracheitis.

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Synthesis of Amino Acids from Urea by the Genus Brucella

H. S. CAMERON, D.V.M., Ph.D., and MARGARET E. MEYER, B.S.

Davis, California

THE OBSERVATION that each of the three species of the genus Brucella possesses the essential enzymes for the synthesis of urea via the ornithine cycle has been reported by Cameron *et al.*[1] and Cameron and Meyer.[2] Brucella organisms also have an active urease enzyme that decomposes urea to carbon dioxide and ammonia.[5] Since both of these substances are essential in many of the various anabolic and catabolic processes of cells, it appeared that one of the functions of the urea cycle, in bacterial cells that contain urease, may be that of supplying ammonia and carbon dioxide to the cell.

This paper, therefore, is a report on investigations to ascertain if these products of urea decomposition by the bacterial cell are used in the synthesis of other substances.

MATERIALS AND METHODS

Suspensions of the resting cells of each species were prepared as described by Cameron and Meyer.[2] Conventional Warburg manometric techniques were used to determine oxygen uptake.[10] The urea was prepared in a concentration of 3 mg. per milliliter in Sorenson's phosphate buffer at a pH of 7.0. Of this concentration 0.5 ml. was used as a substrate to make the total flask contents 3.0 ml. Preliminary experiments were conducted to determine the quantity of urea that would be decomposed by the cells in ten to twenty minutes of incubation. Endogenous rates of oxygen uptake with vessels in which carbon dioxide was absorbed, and controls in which it was not absorbed, were determined for each experiment and exogenous respiration rates were measured at least in duplicate. Further controls consisted of commercially purified urease prepared in gum arabic as prescribed by Sumner[9] and used in a concentration of 10 mg. of urease for each control. The reaction was allowed to proceed for one hour after tipping, at which time it was stopped with sodium azide and the flask contents prepared for chromatographic studies as described by Cameron and Meyer.[2]

In addition to these quantitative studies, cell suspensions with a urea substrate were incubated in a water bath at 37.5 C. for intervals of 24, 48, and 72 hours in order that greater quantities of amino acids would accumulate and allow for more accurate chromatographic identification. The contents of these flasks were identical to those prepared for the Warburg studies.

Ammonia nitrogen was determined by direct nesslerization of the supernatant fluid.

RESULTS

Table 1 shows the comparative rates of urea decomposition and the oxidative utilization of its end products by the three species of Brucella, with commercial urease as the control. It may be seen from this table that the three species of Brucella oxidatively metabolize the decomposition products of urea, provided that the carbon dioxide released by the action of urease upon urea is not absorbed. When the carbon dioxide is absorbed, the oxygen uptake rates are depressed below the endogenous values for *Brucella abortus* and *Brucella suis*, while *Brucella melitensis* showed a low oxidative rate. When the rates are evaluated as the difference between the figure obtained when carbon dioxide is absorbed and when it is not absorbed, the increase in oxygen uptake is significant.

Figure 1 shows the comparative Q Co_2 (N) and Q O_2(N) values both for commercial urease controls and for *B. melitensis* acting upon urea. *Brucella melitensis* was used as being typical of the results ob-

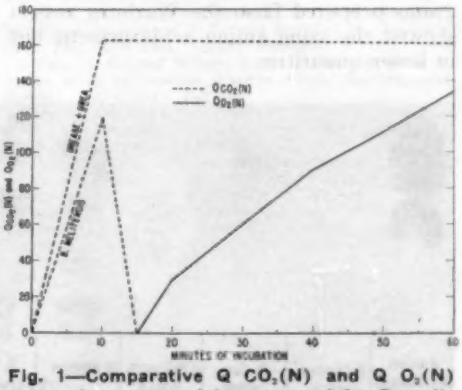

Fig. 1—Comparative Q CO_2(N) and Q O_2(N) values for commercial urease and for Brucella melitensis acting on urea.

From the University of California, Davis.

TABLE 1—Comparative Rates of Urea Decomposition by Urease and Oxidative Utilization of the End Products by the Three Species of Brucella

Source of urease	$QCO_2(N)$*	A $QO_2(N)$* CO_2 absorbed	B $QO_2(N)$* CO_2 not absorbed	C Difference between A and B	Amino acids synthesized
Commercial Urease**	165	No O_2 uptake	No O_2 uptake	.....	None
*B. abortus***	110	−18	45	+63	Glutamic acid and alanine
*B. suis***	90	−50	20	+70	Glutamic acid and alanine
*B. melitensis***	104	+12	125	+113	Glutamic acid and alanine

* All endogenous rates are subtracted; ** substrate consisted of 0.5 ml. of buffer containing 1.5 mg. of urea.

tained for the genus. In the flasks containing the organisms, as well as in the urease controls, there was an immediate release of carbon dioxide during the first few minutes of incubation. With the exhaustion of the substrate during the first ten minutes of incubation, there was then no further activity in the controls. However, with all three species of Brucella there was further activity in that, after twenty minutes of incubation, the carbon dioxide had been utilized and oxygen uptake was occurring in excess of the endogenous controls.

Figure 2 is a photograph of a chromatogram prepared from the supernatant fluid of the vessels that were incubated in the water bath. Controls consisted of cells incubated in buffer without a urea substrate and also commercial urease incubated with urea. This figure shows that leakage of amino acids from the cells would account for only a negligible portion of the amino acids accumulating during the incubation time. The amino acids nearest the base line were arginine and ornithine. Chromatograms prepared from the Warburg vessels showed the same amino acids present but in lesser quantities.

DISCUSSION

Brucella cells decompose urea to form one mole of carbon dioxide and two moles of ammonia.[5] According to Sumner,[9] when urease acts upon urea in the presence of neutral phosphate buffer, the products of the reaction are carbon dioxide, water, and ammonium carbonate. Under the experimental conditions herein reported, in which a phosphate buffer was used, 0.8 mg. of ammonia nitrogen was formed when commercial urease acted upon urea. When Brucella cells decomposed urea, the average ammonia nitrogen per milliliter of supernatant fluid was also 0.8 mg. However, at the end of an hour's incubation of the cells with a urea substrate, the ammonia nitrogen had decreased to an average of 0.65 mg. per milliliter of supernatant fluid. It was evident, therefore, that one of the reactions that occurred during the synthesis of alanine and glutamic acid from urea was the utilization of the ammonia nitrogen that became available from urea decomposition. The amino acid synthesis was also dependent upon the presence of carbon dioxide. By using radioactive $C^{14}O_2$, Marr and Wilson[7] have shown that *B.*

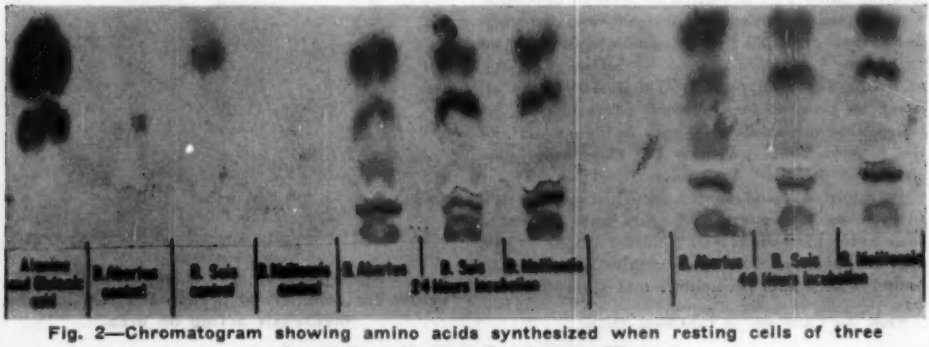

Fig. 2—Chromatogram showing amino acids synthesized when resting cells of three species of Brucella were incubated with urea.

abortus, strain 19, fixes carbon dioxide into the carboxyl group of alanine.

The carbon dioxide initially released by the decomposition of urea was completely used by the cells. It appears, therefore, that fixation of carbon dioxide was involved in the synthesis of alanine and possibly of glutamic acid. The three species are alike in this respect.

Since, according to Hoyer,[5] acetone-dried Brucella cells have about the same degree of urease activity on a weight basis as jack bean meal, a commercial source of urease, it is interesting to observe the role of this enzyme in the physiology of Brucella cells and probably other genera that contain urease. In mammalian physiology, the ornithine cycle functions to synthesize urea from ammonia and thereby detoxifies the ammonia. Gale,[3] in discussing the formation of enzymes, classifies urease under protective mechanisms of the cell, as its substrate is toxic to the organism, inferring, therefore, that urea is toxic to the cells. Ammonia is also toxic to cells. Krebs[6] in discussing the ornithine cycle in Neurospora states that in this organism the cycle is not to be regarded as a method for detoxicating ammonia. Neurospora also possesses urease.[8] The results of the investigation herein reported indicate that in organisms which synthesize urea via the ornithine cycle and contain urease, the functions of these enzyme systems is detoxification by the synthesis of urea from ammonia and protective by the decomposition of urea. Both are necessary adjuncts to one another in the maintenance of an ammonia balance that allows the cell to synthesize amino acids but prevents the ammonia concentration from becoming great enough to become toxic to the cell. Carbon dioxide is also made available to the cell by the action of urease upon urea. The only essential difference between the ornithine cycle in mammals and in bacterial cells is that mammals lack urease and the urea is excreted harmlessly as such. In bacterial cells that contain urease, the urea is decomposed and the metabolites used in the synthesis of new products. On the other hand, organisms such as streptococci do not contain urease and do not synthesize urea via the ornithine cycle. However, they hydrolize arginine to ornithine, ammonia, and carbon dioxide. Gale [4] considers that this enzymatic hydrolysis of arginine provides carbon dioxide essential to growth. Although the enzymatic mechanisms among the bacterial species mentioned are quite different, one of the results of the splitting of arginine is to provide new materials for cellular anabolic activities.

SUMMARY

By use of conventional Warburg and paper chromatography techniques, it has been shown that resting cells of each of the three species of Brucella oxidatively metabolize the decomposition products of urea. The amino acids synthesized from the urea were chiefly alanine and glutamic acid.

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A Comparative Study of the Structure of the Small Intestines of Domestic Animals

CHARLES W. TITKEMEYER, D.V.M., M.S., and M. LOIS CALHOUN, D.V.M., Ph.D.

East Lansing, Michigan

NUMEROUS investigators have found appreciable differences in the structure of the small intestines of various species.

Foust [4] found that there were no plicae circulares in the horse and the dog but that they were present in the ox. In a description of the Peyer's patches in various species, Sisson and Grossman [9] stated that in the horse these patches began about 1 m. from the pylorus and extended to the termination of the ileum. They numbered 80 to 100 in this species while in the ox, they were larger and more prominent and varied greatly in size and number. These same investigators counted 18 to 40 in adult cattle and 20 to 58 in calves. In swine, they estimated that there were 16 to 38 and that these patches began 20 to 50 cm. from the pylorus and extended intermittently to the cecum. Chauveau [1] described an immense "Peyerian gland" in the latter portion of the small intestine of swine, a band measuring 2 m. in length.

Sisson and Grossman [9] found 20 Peyer's patches in the dog. Ellenberger [5] found that these glands in the dog were usually elliptical in outline but the last one was bandlike, reached to the end of the ileum, and was 10 to 40 cm. long in young dogs. Chauveau [1] found only five or six Peyer's patches in the cat.

Numerous species differences in the shape and size of intestinal villi have also been described. Jordan [7] stated that they vary much in form in different mammals and in different portions of the canal in the same individual. He found that they were the most highly developed in the dog where they formed long projections with expanded or clubbed extremities and a constricted base or neck. Foust [4] found that villi were long and slender in carnivores, short and plump in ruminants, and intermediate in horses and swine.

Differences in the musculature of the small intestines have also been described. King and Robinson [8] found that the muscularis mucosae was well developed in the gastrointestinal tract of all mammals and birds, although its thickness was not the same for all species. Vever [10] stated that compared to the intestines of other animals, those of carnivores have thicker and stronger walls. This enables the animal to deal with relatively indigestible flesh and bone which form its diet.

Elias [2] found many differences in the Brunner's glands of various animals. He concluded that there was a great difference in the size of the lumen in the glands of Brunner ranging from approximately 2 μ in the pig to 100 μ in the goat with the horse, cat, ox, dog, and sheep falling between the two in that order.

Johnson [6] made an extensive study of the effects of distention of the intestine. He enumerated these effects as follows: (1) The outer intestinal coats became reduced in thickness. (2) The mucosa became reduced in thickness. (3) The villi became shorter and broader. (4) Glands became shorter and broader. In the guinea pig and mouse they disappeared entirely if the intestine was strongly distended. (5) In the intestine of the guinea pig the epithelium became flattened upon strong distention.

MATERIALS AND METHODS

For this study, the intestinal tracts from 39 mature animals were used. Included in this group were 12 dogs, 7 cows, 7 horses, 7 pigs, 2 sheep, 2 cats, and 2 goats. The intestinal tracts were fixed as soon after death as possible. The intestines of the cows and pigs were obtained from a slaughterhouse where it was impractical to remove them immediately upon the death of the animal. However, they were fixed upon their removal from the carcasses, usually within one-half hour after death. The intestinal tracts of the dogs, cats, and horses were fixed within a very few moments after death. Those of the sheep and goats were obtained from specimens embalmed upon death and were in good condition.

A gross examination of the tract was made before fixation to determine gross appearance of the villi and the number of Peyer's patches. Sections were then taken from the tract every tenth distance irrespective of the over-all length. The first section was taken as near the pylorus of the stomach as possible and the last one was taken next to the ileocecal junction. The other eight sections were taken equidistant between them. These sections were then fixed either in Bouin's solution or in F.A.A. (Lavdowsky's) mixture.[3] After proper fixation, they were dehydrated in dioxane, embedded in paraffin, and sectioned at 8 μ thickness. In order to minimize the effects of distention described by Johnson,[6] sections were taken from areas not distended by fecal material.

Two different staining techniques were used. Hematoxylin and eosin [3] were used on one set of slides to facilitate the study of the villi and the

From the Department of Anatomy, School of Veterinary Medicine, Michigan State College, East Lansing.

Submitted by the senior author in partial fulfillment for the degree of Master of Science, School of Graduate Studies, Michigan State College, East Lansing.

glands. Weigert's and Van Giesen's stains [5] were used on another set as an aid to studying the connective tissue and muscle layers.

RESULTS AND DISCUSSION

Villi.—The gross examination of the intestinal tracts revealed that the villi of the cat were much longer than those of the other species studied. Villi of the dog were next in length, followed in order by those of the goat, sheep, pig, horse, and cow. No attempt was made at measuring the villi macroscopically. Figure 1 shows a macroscopic view of the villi in the duodenum of a dog.

Peyer's Patches.—The presence of Peyer's patches varied considerably in the various species. In the cat, none were observed grossly, but they were found microscopically. This was probably due to their small size. In the horse, the first patch was found only about 1.2 m. from the pylorus indicating that Peyer's patches were located throughout the jejunum and ileum. As the duodenum of the horse is 1.0 to 1.2 m. in length, Peyer's patches began just beyond the terminal end of the duodenum. From this point on they were scattered intermittently throughout the rest of the tract. They were always on the side opposite the mesentery and varied greatly in size and number. In the horses studied, they ranged from 1.5 to 20.0 cm. in length and numbered approximately 85 per animal.

In the cow, Peyer's patches were much larger and more prominent than they were in the horse, but they were less numerous. Upon gross examination, an average of 18 per animal was found. These ranged from 5 to 30 cm. in length and were scattered throughout the jejunum and ileum. The sheep and goats had a few scattered Peyer's patches throughout the jejunum and ileum. In one sheep a Peyer's patch was found extending from the ileum for a short distance into the cecum.

The pigs had about 20 Peyer's patches each. They were prominent and easily distinguishable. The large terminal patch mentioned by Chauveau [1] was not seen.

In the dog, Peyer's patches were easily counted from the exterior surface. They appeared as circumscribed elevations about 2.0 cm. long and 1.5 cm. wide. As a rule they were not directly opposite the mesentery but appeared on either side of it. The number of patches ranged from 17 to 26 with an average number of 22. They were more numerous in the cephalic part of the small intestine than in the ileum. Several patches were found in the duodenum partially covered by the pancreas. They formed a distinct elevation on the interior surface of the tract. Figures 2 and 3 show macroscopic views of typical Peyer's patches in the pig and dog.

Plicae Circulares.—Plicae circulares were found only in the ruminants. The horse, dog, cat, and pig had no circular folds although they did have longitudinal ones running intermittently throughout the intestinal tract. The longitudinal folds of the horse were very prominent but did not assume a circular direction at any level. This is in accordance with the views of Foust [4] that plicae circulares are absent in the horse and dog but are present in the ox.

A microscopic survey of histological sections prepared from various levels of the intestinal tracts revealed numerous variations. Measurements included the height of the villi, the height of the epithelium of the villi, the thickness of the muscularis mucosae, the width of the lumen of the Brunner's glands, the height of the epithelium of Brunner's glands, and the thickness of the muscle layers. A review of the results follows.

The greatest height was found in the intestine of the cat (fig. 4) where the villi had an average height of 963 μ. They were very close together, quite slender, and somewhat constricted at their bases. There was some branching. They were also more numerous than in the other species. The epithelial cells covering the villi were lower in the cat than in any of the other animals studied and reached a height of only 16 μ.

In the dog (fig. 5), the height of the villi was next in magnitude and reached an average height of 645 μ. The villi were numerous and showed some branching. The distal ends were expanded and club-shaped. The height of the covering epithelium was greater than in any other species and reached a height of 32 μ.

The goat had the next tallest villi. They averaged 615 μ in height and were wider at the base than those of the carnivores. They were somewhat expanded at both ends with a slight constriction between. The epithelial layer reached a height of 28 μ.

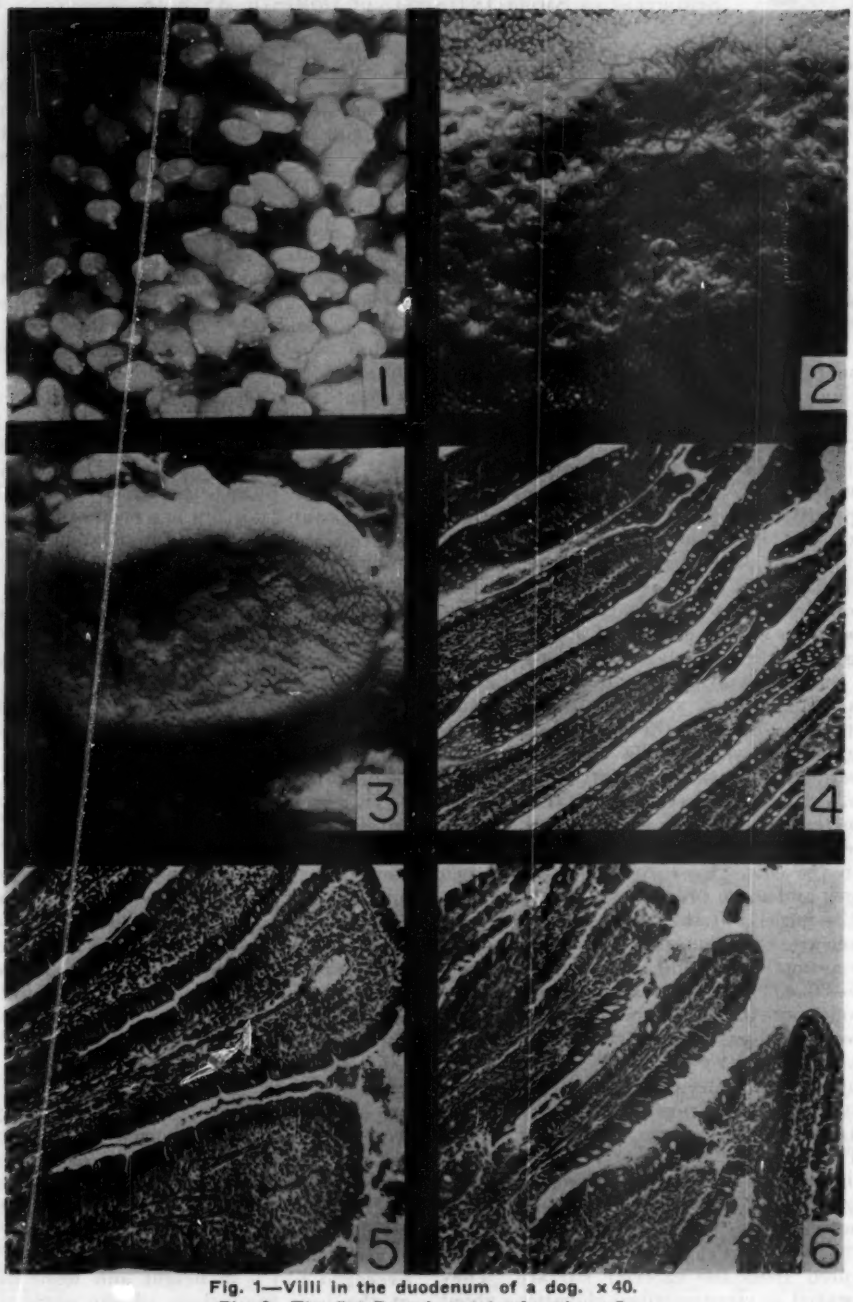

Fig. 1—Villi in the duodenum of a dog. x 40.
Fig. 2—The flat Peyer's patch of a pig. x 5.
Fig. 3—The elevated Peyer's patch of a dog. x 3.
Fig. 4—The tall, slender villi of a cat. Hematoxylin and eosin. x 110.
Fig. 5—Constricted, club-shaped villi of the dog. Hematoxylin and eosin. x 110.
Fig. 6—Widely spaced villi of the pig. Hematoxylin and eosin. x 110.

The villi of sheep and swine were next in length and were nearly equal. Sheep averages totaled 483 μ while those of the hogs totaled 470 μ. In both cases, the villi were cylindrical and were arranged in rows. Those of the swine were less numerous than in the other species studied as shown in figure 6. The height of the epithelial covering was 25 μ in sheep and 29 μ in swine.

The horse had large, broad villi that were well-rounded on their distal ends. The bases were much wider than those of other species. The height averaged 405 μ and the height of the epithelium was 31 μ. In appearance they resembled cylindrical haystacks with a well-formed dome.

The shortest villi were found in the ox. They measured only 363 μ in height and had an epithelial cell covering which measured 22 μ. They were slender and markedly constricted at their bases. They were more numerous than those found in the horse and hog.

Thickness of the Muscularis Mucosae.—In all cases the muscularis mucosae was in two layers, an inner circular and an outer longitudinal one. Except in the dog, these layers were so thin and incomplete it was impractical to measure them separately. The combined layers ranged in thickness from 15 μ in the cat and goat to 26 μ in the horse. In the dog, the muscularis mucosae was markedly different (fig. 7). It was in two layers either of which was thicker than the combined layers in the other species. The inner circular layer averaged 27 μ in thickness; the outer longitudinal layer averaged 44 μ. The two layers totaled 71 μ which is over three times wider than that of the average of other animals such as is shown in the pig (fig. 8).

Brunner's Glands.—Brunner's glands were found in all species but they were much more extensive in some than in others. In the horse, they were found in the first four sections in all cases, and in one animal they were identified in the fifth section. As these sections were taken at every tenth segment of the tract, this indicated that the duodenal glands extended down the tract as far as 9.6 m. from the pylorus.

In the dog, the opposite extreme was found. In 6 of 7 dogs, no Brunner's glands were found in the sections examined. An eighth dog was used and longitudinal sections from various segments of the duodenum were examined specifically for these glands. They were found only in the very proximal part of the duodenum.

In the ox, difficulty was also experienced in locating Brunner's glands. None was found in the sections of the first 6 bovine intestinal tracts examined. A seventh cow was killed and glands were located about 30 cm. from the pylorus. As these glands were found scattered throughout the first 4.0 to 4.5 m. of the duodenum by Sisson and Grossman,[9] it is probable that they were present but were dispersed thinly enough to be missed when sections were taken every tenth distance only.

TABLE 1—Comparisons of the Structure of the Small Intestines of Domestic Animals

	Species						
	Horse	Cow	Sheep	Goat	Pig	Dog	Cat
Number of Peyer's patches	85	18	25	25	20	22	None grossly
Location of Peyer's patches	Jejunum, ileum	Jejunum, ileum	Jejunum, ileum	Jejunum, ileum	Jejunum, ileum	Duodenum, jejunum, ileum	Jejunum, ileum
Plicae circulares	—	+	+	+	—	—	—
Height of villi (μ)	405	363	483	615	470	645	963
Height of epithelium of villi (μ)	31	22	25	28	29	32	16
Thickness of muscularis mucosae (μ)	26	19	24	15	23	71	15
Location of Brunner's glands	Proximal 9.6 m.	Proximal 4.5 m.	Duodenum	Duodenum	Proximal 4.0 m.	Pyloric-duodenal junction	Duodenum
Lumen diameter of Brunner's glands (μ)	7	25	40	50	3	35	20
Height of epithelium of Brunner's glands (μ)	15	12	20	35	25	35	10
Number of layers in tunica muscularis	2	2	2	2	2	3	3
Thickness of tunica muscularis (μ)	1,153	588	287	175	520	701	555

In the other species, duodenal glands were readily found. In the pig they extended at least 4 m. from the pylorus. In the cat, goat, and sheep they were found in the first section only, near the pylorus.

Two measurements were made on the Brunner's glands—the diameter of the lumen and the height of the epithelium. The Brunner's glands of the goat had the greatest lumen diameter while those of the hog had the smallest. The glands of the goat and the dog showed the highest epithelium (fig. 9) while those of the cat had the lowest (fig. 10). Complete measurements are shown in table 1.

The Tunica Muscularis.—The outstanding difference in the muscle layers of the various animals was an extra oblique layer in the carnivores. In the other species, there were distinct inner circular and outer longitudinal layers with an Auerbach's plexus between them. The carnivores had these same two layers plus an oblique layer between the submucosa and the circular layer. In the dog, this layer averaged 47 μ in thickness and was easily

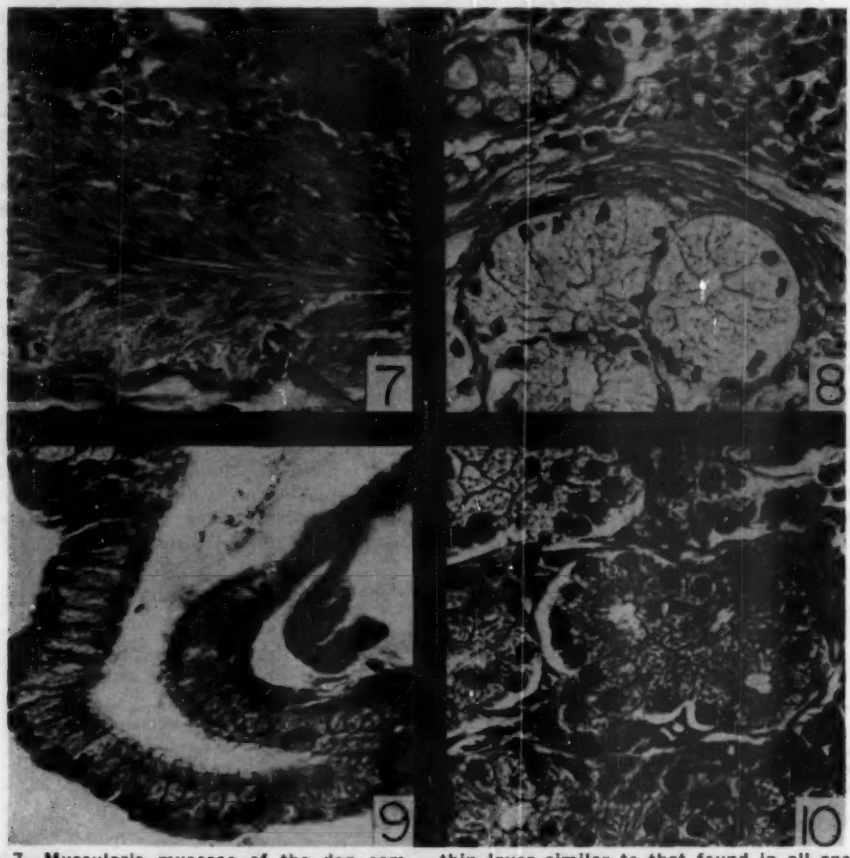

Fig. 7—Muscularis mucosae of the dog composed of an inner circular and an outer longitudinal layer. Between the inner circular layer and the crypts of Lieberkühn is the extra connective tissue layer peculiar to the dog. Hematoxylin and eosin. x 400.

Fig. 8—Muscularis mucosae of the pig, a single thin layer similar to that found in all species except the dog. Hematoxylin and eosin. x 400.

Fig. 9—Brunner's glands of a goat showing the very large lumina and the tall epithelium. Hematoxylin and eosin. x 400.

Fig. 10—Brunner's glands of a cat showing the small lumina and low epithelium. Hematoxylin and eosin. x 500.

identifiable in all sections. In the cat, this oblique layer was only 16 μ in thickness and was difficult to locate in some sections.

The tunica muscularis was the thickest in the horse where it reached an average thickness of 1,153 μ. The longitudinal layer of muscle was nearly as thick as the circular layer. This differed from the other species where the circular layer was much the larger of the two.

Special Connective Tissue Layer.—In the dog, an extra connective tissue layer was observed between the muscularis mucosae and the crypts of Lieberkühn (fig. 7). This zone of connective tissue was about 30 μ in thickness and completely encircled the intestine. It was not observed in any of the other animals studied.

SUMMARY AND CONCLUSIONS

The small intestines of 39 animals representing seven different species were compared grossly and microscopically. A gross examination revealed that the small intestine of the horse contained the greatest number of Peyer's patches. The Peyer's patches of the cat were too small to be seen grossly but were found in microscopic section. Those of the dog were the easiest to identify for they were quite evident as thickened elevations even on the exterior of the intestinal tract. Patches in other animals were flat and could be seen only on the interior surface. Most patches were located opposite the mesentery except in the dog where they usually assumed a position on the lateral and medial sides of the gut tube. They were found in the jejunum and ileum of all species studied and in the dog were found in the duodenum as well.

Intestinal villi were longest in the cat and shortest in the ox. The height of the epithelial cells on the villi was greatest in the dog and least in the cat. No significant difference in the height of the villi was noted at different levels of the tract. Shapes of villi ranged from broad and rounded in the horse to very tall and narrow in the cat. All species except the horse showed a distinct narrowing of the villi at their bases. Villi of the carnivores were expanded on their distal ends. No differences in the shapes of the villi at different levels of the tract were noted.

The muscularis mucosae was very thin in all the species except in the dog where it was found to be in two distinct layers either of which was thicker than the muscularis mucosae in the other species. It was approximately three times thicker than the general over-all average of the other animals studied.

Brunner's glands differed widely both as to extent and form. They were very rare in the dog and were rather thinly dispersed in the cow. They were found in the jejunum of the horse as far as 9.6 m. from the pylorus. In the other species, they were readily identified in the proximal part of the intestine. The diameter of the lumina of the Brunner's glands was greatest in the goat and least in the hog. Epithelial cell height on the glands was highest in the dog and goat and lowest in the cat.

The muscle layer varied considerably. It was the thickest in the horse and thinnest in the goat. The carnivores had an extra oblique layer of muscle between the submucosae and the circular layer. Table 1 shows various comparisons in the species studied.

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The Sterilizing Action of Gaseous Ethylene Oxide on Foot-and-Mouth Disease Virus

A Preliminary Report

MILTON SAVAN, D.V.M.

Lindholm Island, Denmark, and Washington, D. C.

THE BACTERICIDAL action of ethylene oxide has been reviewed by Phillips,[1] and since that time a number of papers have been published reporting its action on a variety of bacteria but little has been published regarding its action on virus material. The preliminary experiments reported here were designed to establish the general range of action of gaseous ethylene oxide on the foot-and-mouth disease virus.

MATERIALS AND METHODS

The procedures and methods employed were essentially those described by Phillips.[2] All glassware and other equipment was kept in the cold room at 4 to 8 C. prior to the experiment and the first steps of the experimental procedure, such as the pipetting of the desired volume of the ethylene oxide into the containers, was also carried out in the cold room. The containers used were 1-liter glass bottles tightly fitted with rubber stoppers and each of the stoppers had a wire hook projecting downward about 18 cm. into the bottle. Pieces of cotton cloth, 2 cm. square, were soaked in the foot-and-mouth disease virus suspension, a 1:1 dilution in phosphate buffer of lymph obtained from vesicles appearing on tongues of cows injected with an "O" strain of the virus, drained and then attached to the hooked wire. They were thus hanging free in the container. The cylinder of ethylene oxide was stored in a deep freeze (—50 C.) but, at the time of the experiment, it was taken into the cold room and the ethylene oxide, then in liquid form, was poured into a slender glass tube. The ethylene oxide was introduced into the liter bottles by removing the stoppers, with their virus soaked cloth squares attached, pipetting in the desired volume of liquid ethylene oxide and quickly replacing the stopper. The bottles were taken from the cold room and kept at room temperature (22 C.) for varying lengths of time before the cloth samples were removed and tested for viable foot-and-mouth disease virus. The cloth samples were tested by placing each in a separate test tube containing 5 ml. of phosphate buffer; the tubes were then shaken for several minutes to insure thorough washing of the cloth and aliquots of the wash fluid were injected intradermally in the footpads of 4 guinea pigs.

RESULTS

In the first experiment, the virus soaked squares were exposed to 1.0, 0.5, 0.05 ml. of ethylene oxide for 30, 60, 120, and 240 minutes and the results given in table 1 indicate that ethylene oxide could effectively destroy the foot-and-mouth disease virus. A second experiment was designed to define more accurately the limits of its action.

The second experiment was modeled after the first except that a lighter type of cotton cloth square was employed and 0.2 ml. of the virus suspension was used to wet the cloth squares rather than the indeterminate amount used in the first experiment. The virus was thus exposed for 5, 15, 30, 45, and 60 minutes at room temperature (22 C.) to 0.1 and 0.05 ml. of ethyl-

TABLE 1—The Reactions in Guinea Pigs Caused by an Indeterminate Amount of Foot-and-Mouth Disease Virus After Exposure to Ethylene Oxide

Exposure time (min.)	Amount of ethylene oxide (ml.)			
	1.0	0.5	0.05	Control*
30	0**	0	+++××	++××
	0	0	0	++×××
	0	0	++×××	++×
	0	0	+++××	++
60	0	0	++	+++××
	0	0	+	+++××
	0	0	+	++
	0	0	+	+++×××
120	0	0	0	++
	0	0	0	+++××
	0	0	0	+++×
	0	0	0	+++×
240	0	0	0	+++××
	0	0	0	+++××
	0	0	0	+++×
	0	0	0	+++××

* Material handled in the same way as the others but ethylene oxide not placed inside the containers.

** Reaction in each guinea pig; 0 indicates no reaction, + indicates the extent of primary lesions, × indicates extent of generalization.

From the State Veterinary Institute for Virus Research, Lindholm Island, Kalvehave, Denmark, and the Animal Disease and Parasite Research Branch, U.S.D.A., Washington, D. C.

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ene oxide. At the end of the various exposure times each cloth square was removed from the ethylene oxide atmosphere and washed in 1.8 ml. of phosphate buffer and the wash material was injected into guinea pigs without further dilution. That portion of the wash material not used in the guinea pig inoculations was stored in the refrigerator at 4 to 8 C. It was thus assumed that each guinea pig was injected with a 1:10 dilution of the virus suspension, since the 0.2 ml., which had been placed on the cloth, was diluted in 1.8 ml. of the wash buffer. The results are presented in table 2. Following the first reading of the lesions on guinea pigs (48 hr. postinjection), the wash material that had been stored in the refrigerator was used in setting up a titration of the residual virus. Only the material from the sixty-minute exposure was used in the titration. The results of these titrations are shown in table 3.

TABLE 2—The Reactions in Guinea Pigs Caused by a 1:10 Dilution of Foot-and-Mouth Disease Virus After Exposure to Ethylene Oxide

Exposure time (min.)	Amount of ethylene oxide (ml.) 0.1	0.05	Control*
5	++**	+	+++×
	+++	0	+++
	+	++	++
	+	+++××	+
	0	++	+++××
15	+++	++	+
	+++	+++××	Died
	++×	+++××	+++×
	++	0	++
	+++××	++	++
30	+	+	+++××
	+	++××	++
	Died	++	++
	+	++	0
	+	Died	+++×××
45	++××	+++××	++
	0	+++××	+++×
	0	++	+++××
	+	++×	++×
	0	+++××	+++
60	++	++	++×
	0	0	++
	0	+×	+++×××
	++	+++××	+++××
	0	0	+

* Material handled in the same way as the others but ethylene oxide not placed inside the containers.

** Reaction in each guinea pig; 0 indicates no reaction, + indicates the extent of primary lesions, × indicates extent of generalization.

TABLE 3—Titration of the Wash Material After Treatment for Sixty Minutes with Ethylene Oxide

Dilution	Amount of ethylene oxide (ml.) 0.1	0.05	Control*
10^{-1}	0**	++×	+++××
	++××	++×	+++××
	+×	+++××	+++
	0	++	++××
10^{-2}	+	0	+++××
	0	++×××	+++×××
	0	0	+++××
	0	++××	+++××
10^{-3}	0	0	+++××
	0	0	++×
	0	0	+++×
	0	0	++××
10^{-4}	0	0	++
	0	0	0
	0	0	++×
	0	0	0

* Material handled in the same way as the others but ethylene oxide not placed inside the containers.

** Reaction in each guinea pig; 0 indicates no reaction, + indicates the extent of primary lesions, × indicates extent of generalization.

SUMMARY

An atmosphere of ethylene oxide produced by 0.5 ml. in a liter container was effective in destroying the guinea pig infectivity of the foot-and-mouth disease virus within thirty minutes, and although complete inactivation of the virus was not attained with the 0.1 ml. of the ethylene oxide, the end point was being approached after exposure for sixty minutes. The virus suspension used was a 1:1 dilution in phosphate buffer of vesicular lymph obtained from cattle experimentally infected with an "O" type of foot-and-mouth disease virus. An atmosphere produced by either 0.1 or 0.05 ml. of ethylene oxide would have been sufficient to completely inactivate a 10^{-3} dilution of the virus suspension within sixty minutes.

Upon the basis of these experiments it is therefore concluded that an atmosphere of ethylene oxide was effective in destroying the guinea pig infectivity of the foot-and-mouth disease virus.

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Chemotherapeutic Properties of Erythromycin Alone and in Combination with Sulfamerazine

H. J. FLORESTANO, Ph.D., and M. E. BAHLER, B.S.

Indianapolis, Indiana

ALTHOUGH considerable information regarding experimental and clinical proof of the efficacy of erythromycin has appeared in the literature, reports pertinent to its veterinary application have been few. Such available publications,[2,3] however, have indicated this antibiotic to be useful in a variety of infections encountered in small animal practice.

Erythromycin, a new antibiotic isolated from a strain of *Streptomyces erythreus*, was first described by McGuire *et al.*[1] The agent was found to possess marked *in vitro* activity against gram-positive organisms, certain large viruses, and rickettsiae. Gram-negative action, however, appeared to be limited to members of the Brucella, Hemophilus, and Neisseria groups; coliforms and other enteric bacilli were highly resistant. *In vitro* and *in vivo* studies also indicated some activity of erythromycin against *Mycobacterium tuberculosis*, *Spirochaeta novyi*, *Endamoeba histolytica*, *Trichomonas vaginalis*, and oxyurids in mice.

Our work with erythromycin has included assay of *in vitro* activity against microorganisms of etiological significance in animal diseases, evaluation of *in vivo* efficacy in experimental mouse infections, and absorption studies in dogs. In view of the growing trend for multiple chemotherapy, the potential value of mixtures of erythromycin with other antimicrobial agents was also investigated. Because of the widespread use of sulfamerazine in small animal medicine, especial interest was given to the study of the behavior of this sulfonamide in combination with the antibiotic.

EXPERIMENTAL DATA

In Vitro Studies.—The sensitivity of various microorganisms to erythromycin was determined by the serial twofold dilution method. Comparison of activity with that of penicillin was made under identical test conditions. Mediums employed for the respective organisms listed in table 1 were as follows: (1) infusion broth* for *Corynebacterium pyogenes*, *Brucella bronchiseptica*, and *Pasteurella boviseptica*; (2) neopeptone (1%)-maltose (4%) broth, adjusted to pH 5.6 for *Candida albicans* and *Trichophyton mentagrophytes*; and (3) brain heart infusion broth (Difco) for all other species studied, with the addition of 5 per cent sterile rabbit blood for optimal growth of *Streptococcus pyogenes*.

Stock solutions of purified crystalline erythromycin and potassium penicillin G (1,550 u./mg.) were prepared in the respective broths to contain 400 μg. of drug per cubic centimeter. Following sterilization through sintered glass filters, subsequent dilutions of the antibiotics were made in broth so that twice the desired final concentration in each tube was obtained in a volume of 0.5 cc. Inoculums of the various bacterial species and of *C. albicans* consisted of 0.5 cc. of a 1:10,000 broth dilution of an eighteen-hour culture of test organism. For *T. mentagrophytes*, growth from Sabouraud's maltose agar after fourteen days' incubation at 28 C. was transferred to flasks containing glass beads and neopeptone-maltose broth, shaken vigorously for ten minutes and strained through cheesecloth. The number of spores was determined by hemocytometer count and the suspension then diluted with broth so that an inoculum of 100,000 spores per cubic centimeter was obtained in the final serial tubes. Antibacterial tests were incubated at 37 C. and examined after twenty-four hours and, finally, after five days, while activity against *C. albicans* and *T. mentagrophytes* was recorded after two weeks' incubation at 28 C.

The antibacterial behavior of erythromycin and sulfamerazine in combination was determined by the same *in vitro* procedure described previously.

TABLE 1—Comparison of In Vitro Activities of Erythromycin and Penicillin

Organism	Minimal complete inhibitory concentration in μg./cc.	
	Erythromycin	Penicillin
Corynebacterium pyogenes	0.024	0.024
Diplococcus pneumoniae (type I)	0.006	0.012
Micrococcus pyogenes var. *aureus* 100	0.391	
Micrococcus pyogenes var. *aureus* 180	0.195	0.024
Streptococcus agalactiae 191	0.195	1.56
Streptococcus agalactiae 193	0.006	0.024
Streptococcus agalactiae 4768	0.006	0.012
Streptococcus agalactiae 6638	0.012	0.003
Streptococcus agalactiae 7077	0.012	0.003
Streptococcus pyogenes	0.019	0.019
Streptococcus uberis	0.391	1.56
Brucella bronchiseptica	6.25	50.0
Pasteurella boviseptica	1.56	0.049
Candida albicans	>500.0	
Trichophyton mentagrophytes	>500.0	

From the Research Department, Pitman-Moore Co., Division of Allied Laboratories, Inc., Indianapolis, Ind.

* Baltimore Biological Laboratory, Inc.

Micrococcus pyogenes var. *aureus* (No. 100) served as the test organism. Each agent was tested alone for end points against the staphylococcus and finally in various combinations as indicated in table 2.

In Vivo Studies.—The therapeutic efficacy of erythromycin was tested against *Streptococcus pyogenes* and *Klebsiella pneumoniae* (type A) infections in mice. Animals weighing 20 Gm. each were infected by the intraperitoneal route so that mice received 1,000 m.l.d. of the streptococcus and 10,000 m.l.d. of *Klebsiella pneumoniae*, respectively. Groups of 10 mice were dosed subcutaneously ten minutes after infection, again at six hours and finally at twenty-four hours following infection. Erythromycin was compared with penicillin against the streptococcal infection and with polymyxin B sulfate in the *Klebsiella pneumoniae*-infected mice. Animals treated as shown in table 3 along with untreated controls were observed for twenty-one days. Those dying within the period of the test were autopsied and heart blood cultured in suitable mediums.

Absorption Studies.—Twenty apparently normal dogs weighing from 7.0 to 19.2 kg. were used. Serum concentrations of erythromycin were determined following oral and intramuscular administration, respectively.

Three different oral forms of erythromycin were given to animals fasted for sixteen hours prior to administration, with adequate rest periods in between dosings: (1) uncoated tablets, each containing 50 mg. of antibiotic, administered at the rate of 10 mg. of drug per kilogram; (2) tablets with a special acid-resistant coating,† containing 100 mg. of erythromycin each, also given at 10 mg./kg.; and (3) uncoated tablets, consisting of 50 mg. of erythromycin in combination with 277 mg. of sulfamerazine, administered so that each dog received 10 mg./kg. of antibiotic and 55.6 mg./kg. (0.39 gr./lb.) of sulfonamide. A fourth tablet (uncoated) of sulfamerazine alone was included for the purpose of comparison of serum sulfonamide levels when administered with and without antibiotic.

† Vetatycin (erythromycin)—trade name of Pitman-Moore Company, Division of Allied Laboratories, Inc., Indianapolis, Ind.

For intramuscular administration, a solution of erythromycin containing 50 mg./cc. was used. The antibiotic was injected in doses of 5 mg. and 10 mg./kg., respectively.

Blood samples were obtained at intervals of 1, 2, 4, and 6 hours following a single dose of each preparation. Erythromycin serum levels were estimated by a serial dilution method described by Ziegler, *et al.*,[4] and serum concentrations of sulfamerazine were determined by the procedure of Bratton and Marshall.[5]

RESULTS

The results of sensitivity tests are presented in table 1. As will be seen, the activity of erythromycin against the gram-positive bacteria was very similar to that of penicillin on a weight basis. Four of the five strains of *Streptococcus agalactiae* showed a more or less uniformity in susceptibility to erythromycin. The exception (strain 191) appeared relatively more resistant to both erythromycin and penicillin. The greater sensitivity of *Streptococcus uberis* to erythromycin than to penicillin is noteworthy. Of the two gram-negative organisms tested, *Pasteurella boviseptica* was the more susceptible to erythromycin. The high resistance of yeast and filamentous fungi reported for erythromycin [6] is exemplified by the data obtained with *C. albicans* and *T. mentagrophytes*.

As shown in table 2, the antibacterial behavior *in vitro* of erythromycin and sulfamerazine in combination against *Micrococcus pyogenes* var. *aureus* was somewhat greater than could be accounted for through mere addition. That the two agents are compatible is indicated by the apparent synergism manifested.

The therapeutic activity of erythromycin in experimental infections in mice is summarized in table 3. The inactivity against the *Klebsiella pneumoniae* infection typi-

TABLE 2—In Vitro Effects of Various Combinations of Erythromycin and Sulfamerazine on Micrococcus Pyogenes var. Aureus (No. 100)

Sulfamerazine (mg./100 cc.)	Activity after 24 hours' incubation: erythromycin (μg./cc.)							
	0	0.009	0.019	0.039	0.078	0.156	0.312	0.625
0	4+*	4+	4+	4+	4+	1+	0	0
0.625	4+	4+	4+	4+	4+	1+	0	0
1.25	4+	4+	4+	4+	4+	0	0	0
2.5	4+	4+	4+	3+	2+	0	0	0
5.0	4+	4+	3+	2+	1+	0	0	0
10.0	2+	2+	2+	1+	1+	0	0	0
20.0	2+							
40.0	0							

* 4+ maximum growth; 1+ to 3+ slight to heavy growth; 0 no visible growth.

fies similar lack of effectiveness of erythromycin against other members of the enteric group of gram-negative pathogens. Against the streptococcal infection, erythromycin was essentially equal in activity to penicillin on a dosage basis.

Serum level measurements of erythromycin showed no wide differences when the antibiotic was administered in the various tablet forms to fasted dogs. The antibiotic was readily absorbed, with greatest variations in serum concentrations occurring during the first two hours. Peak levels usually appeared at one hour following dosage with uncoated tablets of erythromycin, while maximum concentrations occurred at two hours with the specially coated tablets. Subsequent serum levels of antibiotic showed no significant difference, however, regardless of preparation administered. Sulfamerazine concentrations obtained when given in combination with erythromycin paralleled those produced following administration of the sulfonamide alone at the same dosage. Representative serum concentrations of erythromycin and sulfamerazine, when administered simultaneously, are shown in table 4. The serum levels of erythromycin fall in the range of *in vitro* sensitivity of most susceptible organisms. Sulfamerazine concentrations appear somewhat low because of the suboptimal dose (0.39 gr./lb.) employed. For therapeutic levels of this sulfonamide in dogs, the oral administration of 0.5 gr./lb. twice daily is generally required.[7, 8]

Absorption data obtained with single intramuscular doses of erythromycin are given in table 5. The results indicated that effective serum concentrations are provided following parenteral administration of this antibiotic.

DISCUSSION

Data presented here and elsewhere [1, 6, 9, 10] show erythromycin to possess marked antibacterial activity. The antibiotic is effective mainly against gram-positive bacteria, its spectrum being very similar to that of penicillin. *In vitro* results with the various strains of *Streptococcus agalactiae* indicated a uniformity in sensitivity of such strains to erythromycin. A marked consistency in sensitivity of strains of *Micrococcus pyogenes* var. *aureus* to this antibiotic also has been demonstrated.[6]

Regarding the apparent synergism exhibited by the combination of erythromycin and sulfamerazine, it would not be possible to predict such an effect in actual practice on the basis of *in vitro* tests alone. However, it is significant that erythromycin-sulfonamide therapy has been reported to produce definite synergistic effects in mice infected with hemolytic streptococci.[6]

With erythromycin principally active against gram-positive bacteria, its use in

TABLE 4—Representative Serum Concentrations of Erythromycin and Sulfamerazine in Dogs Receiving a Single Dose of Combined Agents in Plain Tablet Form

Hours after dose*	Erythromycin in serum (μg./cc.)		Sulfamerazine in serum (mg./100 cc.)	
	Dog 19	Dog 21	Dog 19	Dog 21
1	1.28	1.28	2.4	2.1
2	0.32	0.64	2.8	2.8
4	0.16	0.32	3.2	3.2
6	0.16	0.16	2.8	2.9

* Dose consisted of 10 mg./kg. of erythromycin and 55.6 mg./kg. (0.39 gr./lb.) of sulfamerazine.

TABLE 3—Therapeutic Activities of Erythromycin, Penicillin, and Polymyxin B Sulfate Against Experimental Infections in Mice

Infecting organism	Antibiotic	Dose* (mg./kg.)	Number of deaths days after infection						Survivors	
			1	2	3	4	5	6-21	(No.)	(%)
Streptococcus pyogenes	Penicillin	5	0	0	0	0	0	0	10	100
		10	0	0	0	0	0	0	10	100
	Erythromycin	5	0	0	0	0	1	0	9	90
	Controls		9	1	0	0	0	0	0	0
Klebsiella pneumoniae (Type A)	Polymyxin B Sulfate	1	0	0	0	0	0	0	10	100
		5	0	0	0	0	0	0	10	100
	Erythromycin	5	9	0	0	0	0	0	1	10
	Controls		7	3	0	0	0	0	0	0

* Respective dose indicated was administered subcutaneously ten minutes following infection, again at six hours, with a third and final dose twenty-four hours after infection.

combination with other antibacterial agents for small animal practice appears warranted. Sulfamerazine may well be considered the drug of choice in this respect

TABLE 5—Serum Concentrations of Erythromycin in Dogs Following Single Intramuscular Administration

Dog (No.)	Dose (mg./kg.)	μg. of erythromycin per cc. of serum—hours after dose			
		1	2	4	6
5	5	2.56	1.28	0.64	0.16
6	5	2.56	2.56	0.64	0.16
16	10	2.56	2.56	1.28	0.32
17	10	2.56	2.56	1.28	0.32

for several reasons: (1) combination with erythromycin would result in strengthened activity against gram-positive organisms; (2) the spectrum would be broadened to include gram-negative forms of pathological significance in animal diseases; (3) a possible synergistic effect is indicated; and (4) sulfamerazine not only produces higher and more sustained serum levels in dogs and eats than any other sulfonamide employed in veterinary practice,[7, 11] but it is also the most active of the long lasting sulfonamides.[12]

Laboratory findings and clinical results obtained thus far with a combination * of erythromycin and sulfamerazine indicate that the two agents are compatible. No interference with absorption of either drug has been observed when simultaneously administered *per os*. The combination is well tolerated, with an emesis rate of about 5 per cent. Whether due to synergism or mere complementary action of its two components, therapeutic response to the mixture has been striking following use in a large number of disease entities in dogs and cats.[13-16]

SUMMARY

1) *In vitro* studies showed erythromycin to possess marked activity against gram-positive bacteria, its action being comparable to that of penicillin on the species tested.

2) Erythromycin was highly effective in experimental streptococcal infection in mice but failed to protect animals infected with *Klebsiella pneumoniae*.

3) Erythromycin administered orally to fasted dogs, either alone or in combination with sulfamerazine, was readily absorbed and well tolerated. A single dose of 10 mg. of the antibiotic per kilogram produced serum levels in the range of *in vitro* sensitivity of most susceptible organisms for at least six hours.

4) Intramuscular administration of erythromycin in doses as low as 5 mg./kg. also resulted in therapeutic serum levels.

5) *In vitro* tests indicated a behavior of erythromycin in combination with sulfamerazine suggestive of synergism.

6) The advantages of a combination of erythromycin with sulfamerazine have been discussed. With erythromycin principally active against gram-positive bacteria, its combintion with sulfamerazine should result in strengthened activity against this group of microorganisms, and a spectrum considerably broadened to include gram-negative forms of etiological significance.

* Meratycin—trade name of Pitman-Moore Company.

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Serological and Physiological Relationships Between Strains of *Vibrio Fetus*

K. E. PRICE, Ph.D.; L. J. POELMA, D.V.M.; JOHN E. FABER, JR., Ph.D.

College Park, Maryland

IN A preliminary report,[1] results obtained with whole cell antigens and prepared rabbit antiserums showed that cross agglutination failed to occur between all strains of *Vibrio fetus*, thus indicating that this species may not be antigenically homologous. It was of interest that seven of the 20 bovine fetal strain antiserums tested were involved in most of the instances where cross agglutination failed to occur. Results also showed that three nonfetal bovine cultures that had been listed as *V. fetus* were serologically unrelated to strains isolated from fetuses.

As a result of this study, the following pathways of investigation were developed:

1) A study of the somatic antigen relationships. This was carried out to determine whether the variations noted in whole cell cross agglutination could be explained on the basis of somatic antigen differences. In the preliminary work cited above, it was reported that only five of the eight strains tested were similar in their cross-agglutination patterns.

2) The selection of a strain that would best meet the requirements of a diagnostic antigen. In the preliminary study, it was shown that only ten strains were agglutinated to high titers by all fetal strain antiserums, so further investigation was limited to these.

3) The biochemical differentiation of bovine strains bearing no serological relationship to *V. fetus* strains. The possibility that such strains are saprophytic and would mistakenly be considered as *V. fetus* on the basis of morphological characteristics lent incentive to this phase of the investigation.

MATERIALS AND METHODS

Cultures.—Fifty strains that had been listed as *Vibrio fetus* were employed in these studies. Thirty-four were acquired from Dr. C. A. Manthei and Dr. A. H. Frank, Animal Disease and Parasite Research Branch, United States Department of Agriculture, Beltsville, Md. Fourteen cultures were obtained at the Live Stock Sanitary Service, College Park, Md. One strain was procured from Dr. W. N. Plastridge, Storrs Agricultural Experiment Station, Storrs, Conn. One strain from the American Type Culture Collection, Washington, D. C., was also included.

Preparation of Antigens and Antiserums.—The preparation of antigens and specific antiserums, as well as the cross-agglutination procedure were with the following exception the same as described in the preliminary report.[1] All strains employed as antigens in somatic agglutination tests were subjected to a two-hour boiling period instead of the thirty-minute period previously employed.

Absorption Technique.—Absorption of somatic agglutinins from rabbit antiserums was carried out by a technique based on one utilized by Kauffman[2] for removal of Salmonella agglutinins. Sixty milliliters of the somatic antigen suspension, previously standardized to contain 450,000,000 cells/ml., were centrifuged for fifteen minutes at 3,500 r.p.m. and the supernatant discarded. This procedure gave a volume of cell mass which could readily be duplicated. Five-tenths milliliter of serum to be absorbed and 0.75 ml. of 0.3 per cent formolized saline, giving a serum dilution of 1:2.5, were added to the tube containing the packed cells and mixed. The tube was incubated for three hours at 37 C. At the end of the incubation period, the tube was again centrifuged for fifteen minutes at 3,500 r.p.m. The supernatant was decanted and placed at —50 C. until tested. On the occasions where it was necessary to reabsorb a particular serum, the 1:2.5 dilution of serum was added to fresh cells in the same proportion as had been utilized for the first absorption.

Determination of Nonspecific Agglutinability.—The titers due to nonspecific agglutinability of *V. fetus* strains in normal cattle serums were determined according to the method utilized by Plastridge *et al.*[3]

Mediums and Reagents for Biochemical Tests.—All mediums were inoculated from a 72-hour thioglycollate broth culture and incubated at 37 C. under 5 to 10 per cent CO_2.

Trypticase nitrate broth (BBL* 155) was employed for the detection of nitrate reduction and indol production. Tubes of the medium were examined after three and five days' incubation.

From the Live Stock Sanitary Service (Price and Poelma), University of Maryland, and the Department of Bacteriology (Faber), University of Maryland, College Park.

* Baltimore Biological Laboratory.

Commercial skimmilk was employed to prepare litmus milk. Five milliliters of a 1 per cent (in distilled water) azolitmin dye was added to each 100 ml. of skimmilk. The medium was sterilized at 121 C. for fifteen minutes. Tubes were examined on the tenth, twentieth, and thirtieth day after inoculation.

Proteolytic ability was determined by inoculation of nutrient gelatin (BBL 155). Cultures were examined after thirty days.

Kligler's iron agar (BBL 170) was selected to determine H_2S production. The medium was inoculated by streaking the surface of the slant and stabbing the butt. The slants were incubated for five days.

Phenol red broth base (BBL 173) plus 10.0 Gm./liter of trypticase (BBL 148) was employed as a basal medium for the determination of acid production in the following 1 per cent substrates: glucose, lactose, sucrose, maltose, xylose, mannitol, sorbitol, and inositol. All tubes were incubated for fourteen days.

Procedures used for the above tests were those described in the Manual of Methods for Pure Culture Study of Bacteria.[4]

RESULTS AND DISCUSSION

A series of cross-agglutination tests employing heat-treated cells and antiserums were used to determine the relationships of *V. fetus* cellular antigens. The results obtained with 16 heat-treated cell suspensions of *V. fetus* and their antiserums are shown in table 1.

Fourteen of the strains employed were of bovine fetal origin. One of the remaining (543) was an ovine fetal strain and the other (D.L.F.) was from bovine vaginal mucus. As was the case with untreated cell suspensions, the highest titers were generally obtained with homologous antiserums.

Agglutination of heat-treated suspensions occurred more readily than did whole cell agglutination, usually being complete in twenty-four hours or less. The agglutinated mass was of granular nature as compared to the more flocculent type of agglutination that occurred with whole cell antigens.

D. L. Fashion (D.L.F.) was serologically unrelated to untreated suspensions of *V. fetus* and also showed no cross agglutination with fetal strain heat-treated cells. The somatic antigen(s) of the ovine fetal strain (543) likewise displayed no relationship to the bovine fetal strains, although it had crossed with untreated suspensions of *V. fetus*.

There was a great deal of variation in the cross reactions of the bovine fetal strains. It was noted that antiserums for strains 2029, 68, D-71, 1875, and 1639 agglutinated some fetal strain antigens to low titers and failed to agglutinate others. When the five strains were employed as antigens, relatively little cross agglutination occurred. These same strains had also produced poor antiserums [1] from untreated cell suspensions. Other strains (7137 and 5748) gave good cross agglutination with almost all of the bovine fetal strains.

Reciprocal absorption tests were carried out with the 14 bovine fetal strains and with 543, the ovine fetal strain. In most cases, a single absorption with a particular strain was sufficient to remove all of its specific agglutinins. When exceptions occurred, absorptions were repeated till no further drop in titer took place. Since, as

TABLE 1—Cross-Agglutination Reactions of 16 Strains of Vibrio Fetus when Tested Against Sixteen Specific Antiserums Prepared from Heat-Treated Cells

	Antiserums															
Antigens	543	2216	R	5748	7137	6359	68	2315	7450	2029	K-5	1875	D-71	8356	1639	DLF	Normal
543	800*	0	0	0	0	0	50	0	0	100	0	50	[illegible]	0	0	0	0
2,216	0	800	400	400	800	400	400	200	400	400	400	400	100	400	100	0	0
R	0	200	800	400	400	400	100	200	400	100	400	100	50	400	100	0	0
5,748	0	400	400	800	400	400	400	400	400	400	400	200	100	400	200	0	0
7,137	0	200	200	800	1600	200	400	200	200	100	200	200	50	400	200	0	0
6,359	0	200	400	400	400	400	200	100	400	100	400	50	50	400	100	0	0
68	50	200	200	400	400	200	800	200	200	400	100	200	0	100	200	0	0
2,315	0	800	400	800	800	400	200	800	400	400	800	200	50	400	200	0	0
7,450	0	800	800	800	800	400	400	400	800	400	800	200	50	800	200	0	0
2,029	50	100	100	200	100	100	200	100	100	800	200	100	0	50	50	0	0
K-5	0	400	400	400	400	200	400	400	400	400	800	50	50	400	100	0	0
1,875	50	200	100	100	100	100	100	50	100	800	100	800	0	100	50	0	0
D-71	0	400	200	400	400	200	50	100	200	50	400	0	400	200	0	0	0
8,356	0	400	400	800	400	800	200	100	200	400	400	200	50	400	200	0	0
1,639	0	200	200	200	200	200	400	200	200	400	100	200	0	50	400	0	0
D.L.F.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Reciprocal of highest dilution giving 4+ reaction.

TABLE 2—Agglutination Reactions (Heat-Treated Cells) Obtained Following Reciprocal Absorption Between 5,748 and 14 Other Vibrio Strains

Antigen	Antiserum (Absorbed by)		Titer	Unabsorbed serum titer
5,748	5,748	(5,748)	0	800*
5,748	5,748	(543)	800	800
543	543	(5,748)	800	800
5,748	5,748	(2,216)	0	800
2,216	2,216	(5,748)	0	800
5,748	5,748	(R)	0	800
R	R	(5,748)	0	400
5,748	5,748	(6,359)	0	800
6,359	6,359	(5,748)	0	400
5,748	5,748	(7,137)	0	800
7,137	7,137	(5,748)	0	1,600
5,748	5,748	(68)	400	800
68	68	(5,748)	0	800
5,748	5,748	(7,450)	0	800
7,450	7,450	(5,748)	0	800
5,748	5,748	(2,315)	0	800
2,315	2,315	(5,748)	0	400
5,748	5,748	(K-5)	0	800
K-5	K-5	(5,748)	0	800
5,748	5,748	(1,875)	400	800
1,875	1,875	(5,748)	0	800
5,748	5,748	(2,029)	400	800
2,029	2,029	(5,748)	0	800
5,748	5,748	(D-71)	800	800
D-71	D-71	(5,748)	0	400
5,748	5,748	(8,356)	0	800
8,356	8,356	(5,748)	0	400
5,748	5,748	(1,639)	400	800
1,639	1,639	(5,748)	0	400

* Reciprocal of highest dilution giving 4+ reaction.

was previously shown in table 1, antiserum prepared from heat-treated cells of strain 5748 agglutinated most of the other strains to fairly high titers, it was decided to employ this strain for initial absorption studies. Each strain was used to absorb a separate aliquot of 5748 antiserum. Absorptions were continued until all the specific agglutinins for the strain being used were removed. Cells of strain 5748 were employed in the same manner to absorb antiserums of the other 14 strains. Table 2 shows the results obtained when these reciprocally absorbed serum aliquots were tested with their homologous antigens. It can be seen that 5748 cells removed all agglutinins from the antiserum of every strain except 543. Reciprocally, 543 cells failed to remove the agglutinins from 5748 antiserum. The fact that the absorptions brought about absolutely no reduction in titer in either case clearly indicates that these strains possess no common somatic antigens. There were eight strains that were capable of removing all the 5748 agglutinins. Since complete reciprocal absorption was obtained in these cases, it must be assumed that the somatic antigens of these eight strains are identical to those found in strain 5748. The remaining five strains failed to remove all the agglutinins from 5748 antiserum, although in most cases some reduction in the agglutinin titer was found.

Additional absorption studies were conducted to determine more clearly the relationship of these five strains to the nine strains possessing identical somatic patterns (type I). Strains 5,748 and 7,137 were selected to represent the type I cells in the study. The results of these absorption tests are shown in table 3. When 5,748 antiserum was absorbed by 5,748 or 7,137 cells, it failed to agglutinate any of the strains being tested. Cells of strains 1,639, 2,029, and 68, however, failed to remove all agglutinins from 5,748 antiserum. These three strains also failed to absorb agglutinins for D-71 from 5,748 antiserum. Since these three strains were similar in every respect, they have been designated type II.

Strain 1,875 failed to remove all agglutinins for types I and II. It also failed to absorb agglutinins for D-71 from antiserum 5,748. This strain will be referred to as type III.

The remaining strain (D-71) has been designated as a fourth type. Although removing some agglutinins for strains of

TABLE 3—Agglutination Reactions Obtained when Seven Heat-Treated Cell Suspensions Were Tested in Absorbed 5,748 Antiserums (Prepared from Heat-Treated Cells)

	Antigens						
Antiserum (Absorbed by)	5,748	7,137	1,639	2,029	68	1,875	D-71
5,748 (5,748)	0	0	0	0	0	0	0
5,748 (7,137)	0	0	0	0	0	0	0
5,748 (1,639)	400*	800	0	0	0	0	400
5,748 (2,029)	400	800	0	0	0	0	400
5,748 (68)	400	800	0	0	0	0	400
5,748 (1,875)	400	800	200	200	100	0	200
5,748 (D-71)	400	800	400	400	400	200	0
5,748	800	1,600	400	400	400	200	400

* Reciprocal of highest dilution giving 4+ reaction.

type I, it did not absorb any agglutinins for types II and III.

The cells of strain 543 are considered as being of still another type (V).

Since types II, III, and IV all contained antigenic fractions found in type I cells, cells from a representative strain of each of these three types were employed to absorb an antiserum prepared against a type I strain (7137). When this absorbed serum was tested with 7,137 antigen, the resulting titer was 1:200. Although a marked lowering of the unabsorbed serum titer had occurred (1:1,600 to 1:200), all the agglutinins could not be removed by these types.

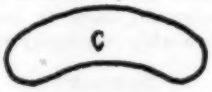
On the basis of these results the following schematic representation is proposed:

Type I—agglutinins completely absorbed by type I only:

Type II—agglutinins completely absorbed by types I and II:

Type III—agglutinins completely adsorbed by types I, II, and III:

Type IV—agglutinins completely absorbed by types I and IV:



Type V—agglutinins completely absorbed by type V only:

If we arbitrarily designate the antigens of type I strains A, B, C, and X, then the three type II strains must contain part but not all of the fractions found in type I. If we assume that they contain A and B, then the third type must contain A or B since the type II cells will absorb all the agglutinins from type III antiserums and reciprocal absorption does not occur. The fourth type must contain a fraction found in type I but not in types II and III. This fraction, according to our scheme, must be C. Since the antigen(s) of the fifth type are not found in type I cells the letter Y has been employed to represent its cellular antigen(s). The remaining fraction or fractions in type I cells have been called X. The presence of this X fraction is verified by the fact that removal of agglutinins for the A, B, and C fractions failed to exhaust the antiserums of type I strains.

It should be noted that the five bovine fetal strains that represent types II, III, and IV were the same five strains that produced poor antiserums from both untreated and heat-treated cell suspensions. Apparently the smaller number of antigenic components found in these cells played a direct role in the amount of activity shown by the specific antiserums. Since these strains produced fairly good agglutinating antigens, it would appear that the agglutinability of the cells was not influenced as markedly as was the antigenicity.

Since the ovine strain (543) shows close relationship to the bovine fetal strains in whole cell cross-agglutination tests, despite the fact that it has no somatic antigen in common with these strains, a possible common flagellar antigen(s) is indicated.

Future work may clarify the role that flagellar antigens play in whole cell cross-agglutination tests. Until then it will be necessary to select strains for the vibrionic agglutination test on the basis of results obtained when strains being considered as antigen possibilities are tested against a large number of specific whole cell antiserums.

In the preliminary report,[1] it was noted that only ten of 30 related strains were agglutinated to high titers by all fetal strain antiserums. These ten antigens were then considered to have the greatest potentialities as diagnostic antigens. It was assumed at this time that the specificity of agglutinins produced in cattle serums was the same as that observed in rabbit serums. Subsequent work [5] has shown this assumption to be true.

Studies employing a limited number of bovine serums from known cases of vibri-

osis have shown that, of the strains tested, 175, 11, and R-2 were the most sensitive to *V. fetus* agglutinins. These three strains were agglutinated at dilutions of 1:400 or higher by almost all positive serums tested. A determination of the nonspecific titer obtained with normal bovine serums indicated that strains 11 and R-2 were agglutinated at dilutions of 1:100, while strain 175 agglutinated at a dilution of only 1:50 in normal serums. On the basis of these results, it appeared that 175 would be the strain of choice. This strain was agglutinated to a titer of 1:400 by 12 of the 13 known positive bovine serums and to 1:200 with partial agglutination in the 1:400 by the remaining serum. On the basis of the above findings, the following dilutions were selected for routine diagnostic tests:

> complete or partial agglutination in 1:100 = negative; complete agglutination in 1:100 and partial in 1:200 = suspicious; complete agglutination in 1:200 or higher = positive.

Extensive field trials will be necessary to show the value of strain 175 as a diagnostic antigen; however, it is believed that this is as satisfactory an antigen as can be obtained at this time.

Three of the ten bovine strains isolated from sources other than the tissues or stomach content of aborted fetuses showed no serological relationship to fetal strains.[1] This lack of serological relationship would indicate that these strains are not *V. fetus*, although almost identical in their morphological characteristics.

Due to the impracticability of distinguishing these strains from *V. fetus* by serological means, the possibility that there might be some physiological differences was investigated. Fifty strains, which included 33 that had been previously studied serologically,[1] as well as 17 bovine strains whose serological relationships had not been studied up to this time, were subjected to biochemical studies. The 30 known *V. fetus* strains and the 17 serologically untested strains were found to have the following characteristics: unable to grow in gelatin; reduced litmus milk (when growth occurred); indol negative; nitrate positive (1 exception); H_2S negative; and no acid from the carbohydrates and polyalcohols tested. Comparison of these results with those given in the sixth edition of Bergey's manual[6] for this species showed that the results obtained with gelatin, indol, and carbohydrates were the same. However, *V. fetus* was characterized as being unable to grow in litmus milk, while the results for nitrate reduction and H_2S production were unreported. The fact that *V. fetus* is nitrate positive would result in its being shifted in the "key to the species of genus Vibrio" from II C (nitrate not reported) to II A (nitrites produced from nitrates).

The three serologically unrelated strains were similar to *V. fetus* in every characteristic except H_2S production. When inoculated in Kligler's iron agar, these three strains were found to produce almost complete blackening of the medium after three days' incubation at 37 C. The possibility of employing H_2S production as a means of differentiating such strains from *V. fetus* was noted.

On the basis of their inability to produce H_2S, the 17 serologically untested strains should be designated as *V. fetus*. To prove this assumption, cell suspensions prepared from these 17 strains were tested against five specific *V. fetus* antiserums. Cross agglutination was obtained with all antigen-antiserum combinations indicating that the 17 strains were serologically related to *V. fetus*. Additional strains will have to be tested to establish whether this procedure can satisfactorily differentiate all strains from bovine sources that are serologically unrelated to *V. fetus*.* Also, investigations should be conducted to determine whether these unrelated strains are capable of causing abortion. The fact that no such strains were found in actual cases of abortion would seem to indicate that these are probably saprophytic species.

SUMMARY

Results obtained from cross-agglutination and reciprocal absorption tests, using heat-treated cell suspensions, indicated that the somatic antigens of the bovine strains were of four different types and these were designated as types I, II, III, and IV. One ovine strain, serologically unrelated to the 14 bovine strains, was designated as type V.

Studies employed to select a strain to be used as a diagnostic antigen showed that

* Fifteen serologically unrelated strains of Vibrio isolated from nonfetal sources by Bryner and Frank[7] of the Animal Disease and Parasite Research Branch, U.S.D.A., Beltsville, Md., were all found to be H_2S positive.

three of the potential diagnostic antigens were highly sensitive to *Vibrio fetus* agglutinins in cattle serums. Strain 175 was found to be the most satisfactory of these since it gave the lowest nonspecific titer in normal bovine serums.

Biochemical studies revealed that all strains tested were indol negative, nitrate positive (1 exception), and failed to grow in gelatin. None of the strains produced acid in basal mediums containing various carbohydrates and polyalcohols. Most were found to bring about some reduction of litmus milk. Three serologically unrelated strains were the only H_2S producers.

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The Influence of Age of Human-Type Tubercle Bacilli Cultures on the Potency of the Culture Fluids

L. A. BAISDEN, Ph.D.; A. B. LARSEN, D.V.M., M.S.;
T. H. VARDAMAN, D.V.M., M.S.

Beltsville, Maryland

THE INFLUENCE of age of cultures of the strains of human-type tubercle bacilli, used in the laboratories of the Animal Disease and Parasite Research Branch (ADP) of the Agricultural Research Service, U.S.-D.A., for tuberculin production, upon the potency of the culture filtrates as measured by the reactions resulting from injecting them intradermally into cattle was studied in two experiments carried out in 1948 and 1949. However, the publication of the results has been delayed in the hope that further experiments could be carried out along these lines. It appears unlikely at

From the Animal Disease and Parasite Research Branch (Baisden) of Agricultural Research Service; from the regional laboratory (Larsen), Auburn, Ala.; formerly with regional laboratory (Vardaman), Auburn, Ala.

TABLE 1—Influence of Age on Skin Activity of Human Tubercle Bacilli Culture Filtrates, Experiment 179

Material	Bovis-Sensitized Cattle			Paratuberculosis-Sensitized Cattle		
	Dose (O.T.)	Ave. reaction (mm.)	Potency ratio	Dose (O.T.)	Ave. reaction (mm.)	Potency ratio
Standard	1/40	10.37		1/10	11.12	
	1/160	6.96		1/40	5.31	
	1/640	4.36		1/160	3.29	
	1/2560	2.83	1.0	1/640	2.07	1.0
2-week culture filtrate	1/10	10.26		1/10	4.71	
	1/40	6.18		1/40	2.89	
	1/160	3.98		1/160	1.57	
	1/640	1.89	0.19*	1/640	0.78	0.15*
2-week culture intradermic strength	1/4	13.15		1/4	6.09	
	1/16	8.96		1/16	3.84	
	1/64	5.46		1/64	2.57	
	1/160	2.73	0.19*	1/160	1.01	0.11*
5-week culture filtrate	1/100	9.36		1/25	5.55	
	1/400	6.80		1/100	4.04	
	1/1600	3.79		1/400	1.92	
	1/6400	2.31	1.82	1/1600	1.15	0.61
5-week culture intradermic strength	1/40	10.85		1/10	8.46	
	1/160	7.11		1/40	5.20	
	1/640	4.55		1/160	3.07	
	1/2560	2.73	1.10	1/640	2.07	0.66
8-week-culture filtrate	1/100	11.45		1/25	8.43	
	1/400	8.18		1/100	5.10	
	1/1600	4.10		1/400	2.97	
	1/6400	2.13	2.95*	1/1600	1.29	1.49
8-week culture intradermic strength	1/40	10.17		1/10	8.17	
	1/160	8.03		1/40	5.28	
	1/640	3.88		1/160	2.84	
	1/2560	2.30	1.00	1/640	1.97	0.62
11-week culture filtrate	1/100	11.76		1/25	8.1[illegible]	
	1/400	7.96		1/100	4.71	
	1/1600	4.38		1/400	3.00	
	1/6400	2.34	2.50*	1/1600	1.16	1.32
11-week culture intradermic strength	1/40	10.89		1/10	7.70	
	1/160	7.58		1/40	3.99	
	1/640	3.98		1/160	2.30	
	1/2560	2.21	1.00	1/640	1.01	0.40*

* Significantly different from standard, a difference in potency ratio of 1.9 times being significant for the comparisons on the bovis-sensitized cattle, and of 2.3 times on the paratuberculosis-sensitized cattle.
O.T.—Old Tuberculin.

this time that further studies on this subject will be made in the near future.

Sandor[1] and Seibert and Munday[2] have studied the influence of age of cultures of tubercle bacilli on the amount of precipitable protein in the culture filtrates. Certain laboratories have established the age for harvesting of cultures at six weeks for tuberculin production and for the isolation of various products of growth. Others, including those of the Bureau of Animal Industry have established the period of incubation at eight or twelve weeks before harvesting of the cultures. Tuberculin is now produced in the laboratories of the ADP.

Experimental Data.—Cultures of the ADP human-type tubercle bacilli, strain Pn in experiment 179, and strains Pn, C, and Dt in experiment 183 were grown on the Henley[3] synthetic medium for the desired time interval and then killed by heating in flowing steam for three hours. The growth was strained off and the culture fluids filtered through double thicknesses of filter paper. The culture filtrates of experiment 179 were divided into two portions. One portion, identified as "culture-filtrate" in table 1, was tested at the appropriate dilutions. The second portion, identified as "intradermic strength," was evaporated before testing. The culture filtrates of the three strains of experiment 183 were combined for test, unevaporated.

The tests were carried out using an incomplete block experimental design mentioned by Wadley[4,5] which called for testing 16 materials (the materials discussed here being among them) at four concentrations each on each of 10 cattle sensitized with a mineral oil suspension of heat-killed *Mycobacterium tuberculosis* var. *bovis* and 10 cattle similarly sensitized with *Mycobacterium paratuberculosis.*

The responses were measured in millimeter increases in skin thickness resulting from the injections of 0.2-ml. amounts of appropriate dilutions of the materials.

As a standard in experiment 179, equal amounts of five regularly produced ADP intradermic tuberculins were pooled; all tuberculins were less than 1 year old. This standard was used at the concentrations indicated in table 1. The standard in experiment 183 was a pool of 14 ADP intradermic tuberculins, all less than a year old (table 2). The concentrations used were in terms of Old Tuberculin which is considered as unit strength when it is made by evaporating the culture fluid to 10 per cent of its original volume. The intradermic strength of ADP tuberculins, and of the intradermic-strength filtrates used here, were one fourth Old Tuberculin strength, as produced, since the culture fluid was evaporated to 20 per cent of its original volume and then diluted back to 40 per cent with a 1 per cent phenol solution. Appropriate dilutions of these preparations were made for the tests. The culture filtrates used here were one

TABLE 2—Influence of Age on Skin Activity of Human Tubercle Bacilli Culture Filtrates, Experiment 183

	Bovis-Sensitized Cattle			Paratuberculosis-Sensitized Cattle		
Material	Dose (O.T.)	Ave. reaction (mm.)	Potency ratio	Dose (O.T.)	Ave. reaction (mm.)	Potency ratio
Standard	1/16	11.48		1/4	10.57	
	1/64	6.37		1/16	6.52	
	1/256	3.98		1/64	3.56	
	1/1024	1.61	1.00	1/256	1.66	1.00
2-week culture filtrate	1/10	3.74		1/10	2.68	
	1/40	2.26		1/40	0.97	
	1/160	1.41		1/160	0.80	
	1/640	0.81	0.05*	1/640	0.46	0.02*
3-week culture filtrate	1/10	6.96		1/10	3.88	
	1/40	4.71		1/40	2.30	
	1/160	2.84		1/160	1.23	
	1/640	1.15	0.23*	1/640	0.61	0.25*
4-week culture filtrate	1/10	8.50		1/10	5.47	
	1/40	5.04		1/40	3.17	
	1/160	2.78		1/160	1.96	
	1/640	1.50	0.31*	1/640	0.71	0.47*
6-week culture filtrate	1/40	9.13		1/10	7.40	
	1/160	4.65		1/40	3.72	
	1/640	2.61		1/160	2.45	
	1/2560	1.25	1.26	1/640	1.23	0.89
11- to 12-week culture filtrate	1/40	8.74		1/10	6.15	
	1/160	5.00		1/40	3.81	
	1/640	2.80		1/160	1.55	
	1/2560	2.13	1.55	1/640	0.82	0.60

* Significantly different from standard, a difference of 2.0 times being required on bovis cattle, and of 1.5 times on paratuberculosis cattle.

O.T.—Old Tuberculin.

tenth Old Tuberculin strength before dilutions were made, since, of course, these filtrates were subjected to no concentrating process.

RESULTS AND DISCUSSION

The results are given in tables 1 and 2. The standards are assumed to show unit potency which is expressed as potency ratios of 1.0.

It can be seen that, in general, the potency ratios (potency as compared to the standard) of the culture filtrates and of the intradermic-strength filtrates increase with the age of the culture. The increase is more consistent on the bovis-sensitized cattle than on the paratuberculosis-sensitized cattle.

In addition to the main conclusion to be drawn from these data, two other points of interest may be suggested for consideration. The first of these points is that in experiment 179 the older unevaporated culture filtrates are more potent than their evaporated partners (intradermic strength) on the basis of their Old Tuberculin strengths. There is also some indication that they are slightly more potent than the evaporated ones on the basis of the concentrations that would actually be used in field testing, *i.e.*, one tenth Old Tuberculin strength for the unevaporated culture filtrates and one fourth Old Tuberculin strength for the evaporated ones. Although a strict test of this point is not made in experiment 183, the results indicate that the older unevaporated culture filtrates might possibly not compare so favorably with the evaporated preparations as in the earlier experiment. It would appear desirable to make further comparisons of the potencies of the two types of preparations under both laboratory and field conditions. Use of unevaporated tuberculins would increase the capacity of a production unit by two and one half times. The ADP laboratories produce approximately 1,100 liters of intradermic tuberculin per year at present.

A second point worthy of attention which is shown in the results of these experiments is that a five- or six-week incubation period would probably be sufficient in case of an emergency.

SUMMARY

In two experiments, it has been shown that the potency of tubercle bacillus culture fluid increases with age of culture; in one experiment unevaporated culture fluid from cultures of the usual age for harvest was as potent as evaporated culture fluid.

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Polysaccharide Antigens from Three Leptospiral Serotypes

MORRIS D. SCHNEIDER, D.V.M., Ph.D.

Fort McPherson, Georgia

IN 1928, the Rockefeller Institute for Medical Research had directed a commission to investigate an outbreak of yellow fever in the Gold Coast, West Africa. At that time, the identity of the etiological agent of this disease was still a controversial subject. Dr. Hideyo Noguchi, a member of the commission, had isolated cultures of leptospiras from cases of epidemic jaundice, erroneously diagnosed "yellow fever," and had named the agent *Leptospira icteroides*.[1] However, his mistake about the etiology of this disease cost him his life. Noguchi's investigations of the leptospiras have served as a stimulus to many microbiologists, and his work on the development of immunological techniques for distinguishing serotypes of leptospiras remains a classic.[2] There are many articles on the significance of these spirochetes [3-5] * but knowledge of their basic chemical constitution is rather meager.[4,6] An insight into their pathogenic behavior, through their chemical composition, would provide us with a rational approach to chemotherapy and prophylaxis. Their classification depends upon the use of agglutination-lysis reactions completed by cross-absorption tests of hyperimmune serums with suspensions of cross-reacting leptospiras.[7] Taxonomists need improved techniques for determining the antigenic pattern of leptospiral organisms, requiring studies which deal with their chemical constitution, immunochemical characteristics of cell-free extracts, and their metabolism. It is not inconceivable that a well characterized, cell-free, antigenic and immunogenic extract of the leptospiral cell will be prepared which will eliminate the laborious absorption techniques now employed for separating group reactive antibodies.

The purpose of this communication is to give an account of the chemical constitution and complement-fixing properties of one of several cell-free components which has been isolated from selected serotypes of leptospiras.[8-11] The aqueous cell-free extract, designated as fraction 1, reacts in the complement-fixation test preferentially with homologous hyperimmune serum and to a lesser extent with hyperimmune serums prepared against other serotypes of leptospiras. The evidence relating to the identity of the major complement-fixing antigen system contained in fraction 1 is summarized in this report.

MATERIALS AND METHODS

By techniques already described,[8,10,11] preparations of fraction 1 were obtained from *Leptospira icterohemorrhagiae* AB (Wijnberg), *Leptospira canicola*, and *Leptospira bataviae*.† A brief description of the fractionation method is as follows: Culture-propagated viable leptospiras were collected by centrifugation, washed twice, and resuspended in phosphate buffered saline solution at pH 7 in a volume equal to 2 per cent of the original culture. The cells were disintegrated with an equal volume of disrupting fluid containing 0.1 M NaCl, 0.2 M sodium citrate, and 0.4 per cent sodium desoxycholate. The suspension was shaken with one-half volume of a mixture of chloroform (4 parts) and normal amyl alcohol (1 part) and centrifuged. The preparation separated into (*a*) an aqueous phase, (*b*) an interfacial cell-protein gel, and (*c*) a chloroform layer. The aqueous phase yielded, on further processing by pervaporation and dialysis in a Visking cellophane sac against water or saline solution, a preparation in a volume equal to 4 to 6 per cent of the culture volume. The latter nondialyzable aqueous material has been designated "fraction 1." For a more detailed account of the fractionation technique *see* references cited.

The preparations were examined chemically by

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The writer is indebted to the Departments of Veterinary Bacteriology and Veterinary Clinical Pathology, Army Medical Service Graduate School, Walter Reed Army Medical Center, for supplying the leptospiral suspensions and the hyperimmune serums and for the performance of complement-fixation tests.

* A comprehensive review of the significance, epidemiology, morbid anatomy, and clinical manifestations of leptospirosis in animals and in man is presented in papers from a Symposium on the Leptospiroses in December, 1952, at the Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C.

† Strains are from the Leptospira Type Collection of the Veterinary Division, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C.

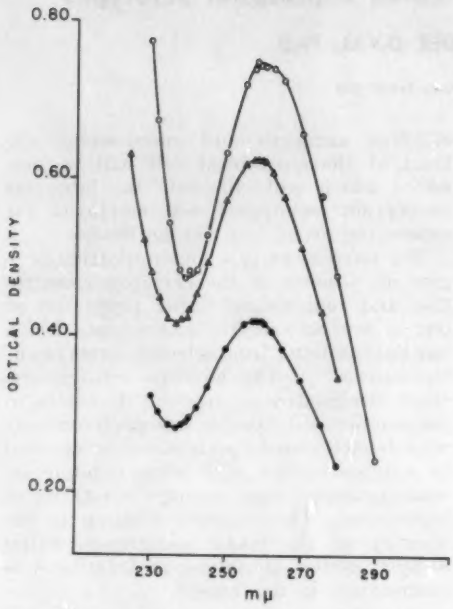

Fig. 1 — Ultraviolet absorption spectrums of each of three representative preparations of fraction 1 of Leptospira icterohemorrhagiae. The strong absorbance in each of the curves seen at the wave length of 258 mμ is confirmatory evidence for the presence of nucleic acids in these preparations.

semimicroanalytical procedures and serologically by means of a semiquantitative complement-fixation reaction.

RESULTS

Chemical Composition.—Fraction 1 is a uniform, remarkably stable, finely divided colloidal suspension containing approximately 40 per cent of the dry weight of the leptospiral cell. It is a macromolecular material and will not pass through the wall of a cellophane sac when dialyzed against water, physiological saline, or phosphate buffered saline solution at pH 7. In the aqueous phase, when stored at 3 to 5 C., fraction 1 has retained its full reactivity as a complement-fixing antigen for longer than a year. Qualitative chemical tests have disclosed that unhydrolyzed preparations fail to give biuret or ninhydrin reactions. However, trace protein contaminants have been detected by means of a sensitive alkaline-copper-Folin reagent.[12] Fraction 1 reacts strongly in the Dische test for carbohydrate. Tests with an orcinol-sulfuric acid reagent indicated that preparations contain about 25 to 30 per cent carbohydrate (by weight) measured as glucose. A partial chemical analysis shows that fraction 1 is composed of approximately 7 per cent organically-bound nitrogen, 1 per cent phosphorus, 8 per cent pentose, 7 per cent methylpentose, 3 to 4 per cent hexose, and about 8 per cent desoxyribonucleic acid. Significant chemical differences have not yet been demonstrated in similar preparations of fraction 1 of three serotypes of leptospiras.

Nucleic Acids.—Figure 1 illustrates the ultraviolet absorption spectrums of prepa-

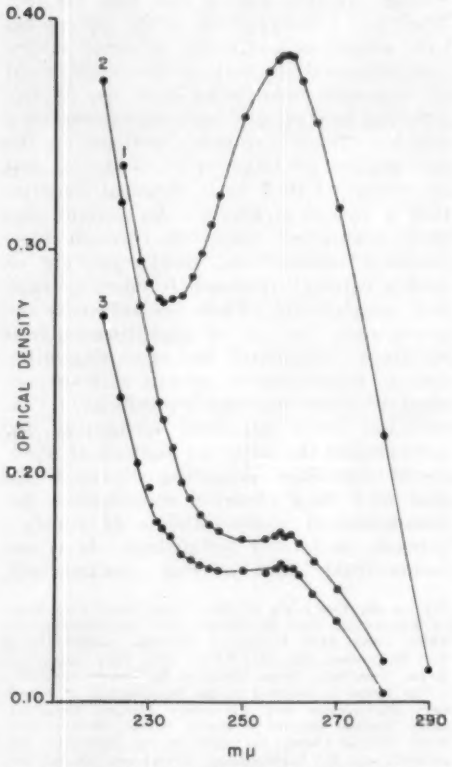

Fig. 2—Specific absorptivity of a preparation of fraction 1 of Leptospira icterohemorrhagiae at 250 mμ (curve 1) falls after treatment simultaneously with crystalline ribo- and desoxyribonuclease followed by dialysis (curve 2). Curve 3 is the second curve minus the optical density of the enzyme blank.

rations of fraction 1 of *L. icterohemorrhagiae*. A strong absorbance occurs at the wave length of 258 mμ which is confirmatory evidence for the presence of nucleic acids. The characteristic specific absorptivity falls upon treatment of fraction 1 with crystalline preparations of ribo- and desoxyribonuclease followed by dialysis. In a typical experiment, the absorbance at 258 mμ of an aqueous cell-free extract of *L. icterohemorrhagiae* was found to be 1.020. After hydrolysis with crystalline ribonuclease followed by dialysis, the absorbance of the residue material inside the dialysis sac had fallen to 0.528 and, following treatment with crystalline desoxyribonuclease, to 0.432. The optical density of a similarly treated control, in the absence of either enzyme, at the end of the experimental period, read 1.040. The ultraviolet absorbing residues appeared in the dialysates of the enzyme-treated material, thus qualitatively confirming the presence of both types of polynucleotide in fraction 1. In an experiment in which fraction 1 was treated simultaneously with both enzyme preparations, the absorbance at 258 mμ was virtually eliminated as is shown in figure 2. Since, after each experiment, all of the active complement-fixing principle remained nondialyzable, the polynucleotide moiety was virtually eliminated as the possible determinant of complement-fixation reactivity.

Pentosan Polysaccharide.—The constituent monosaccharides of fraction 1 preparations have been studied by means of ultraviolet spectroscopy. Samples of fraction 1

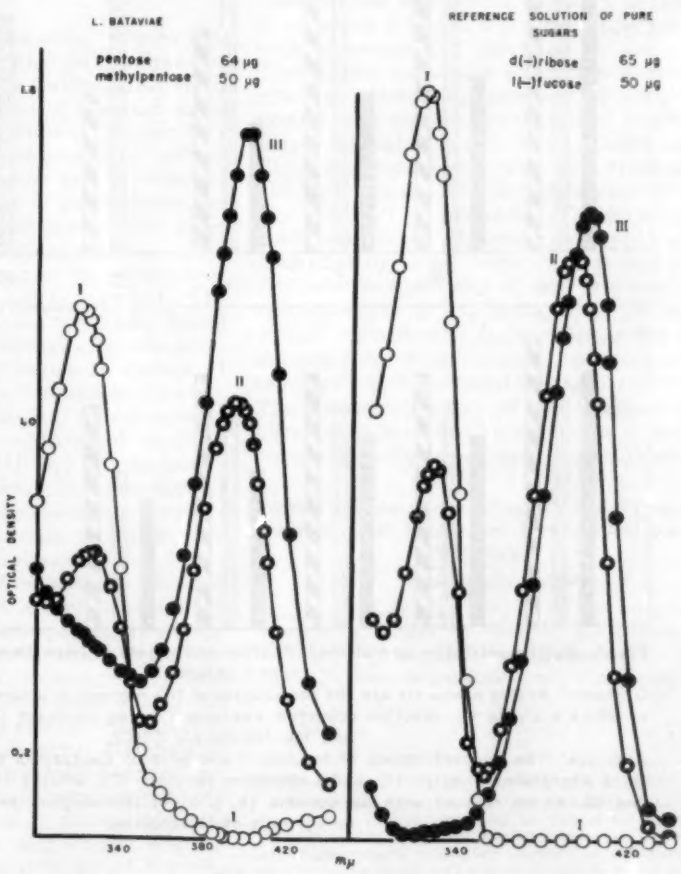

Fig. 3 — Characteristic absorptivities of reaction mixtures of fraction 1 antigen of Leptospira bataviae and a reference solution of pure sugars with sulfuric acid were determined in a Beckman model DU spectrophotometer. The spectrums before the addition of cysteine are shown in curves I.

Primary color complexes found fifteen to forty-five minutes after addition of cysteine are seen in curves II and are characteristic of pentoses.

Secondary reaction products observed twenty-four hours after the addition of cysteine are illustrated in curves III and are characteristic of methylpentoses. The spectrums were measured against a reagent blank containing water in place of the sugar samples. The amounts of pentoses and methylpentoses indicated in the figure were calculated from data obtained with d(—)ribose and l(—)rhamnose.

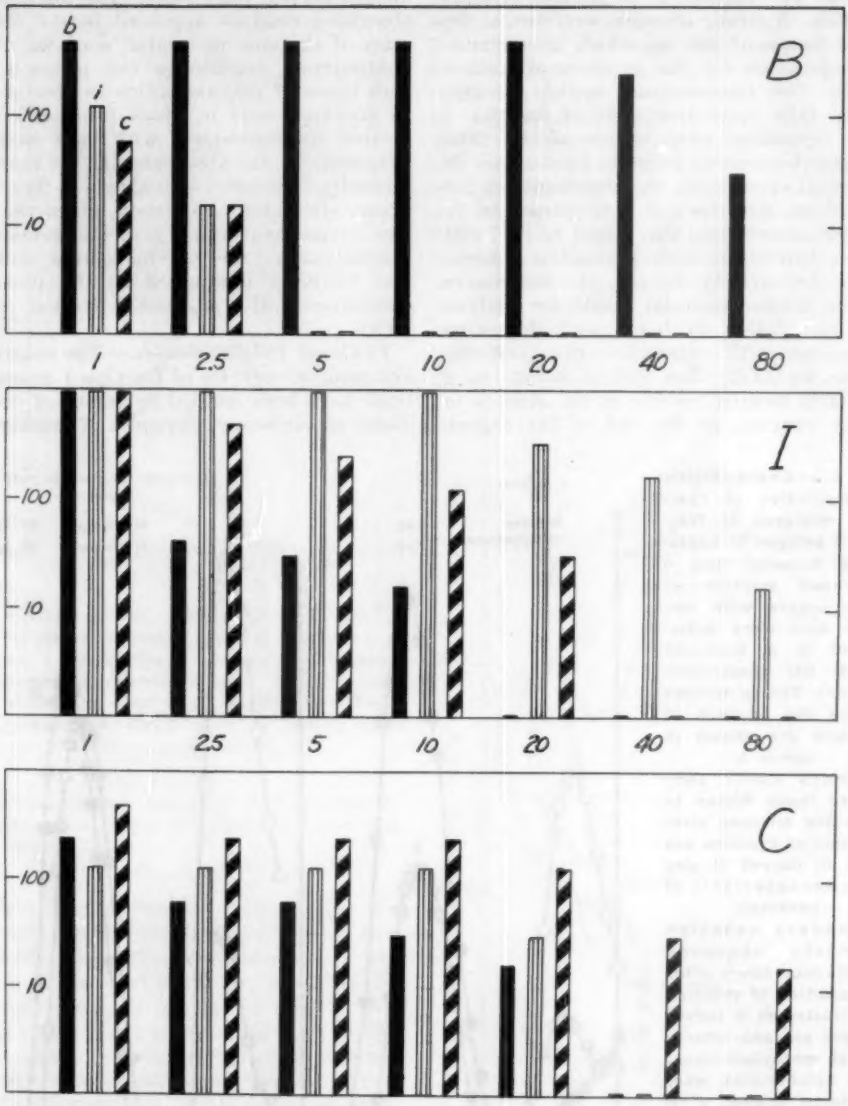

Fig. 4—Semiquantitative complement-fixation end points of hyperimmune, antileptospiral rabbit serums.

Ordinate: Arabic numerals are the reciprocals of the highest dilutions of immune serums at which a 2+ to 4+ reaction occurred, expressed as log numbers (log 10 equals 1.000; and log 100 equals 2.000).

Abscissa: The concentrations of fraction 1 antigens of Leptospira bataviae (B), Leptospira icterohemorrhagiae (I), and Leptospira canicola (C), diluted 1: 1, 2.5, 5, 10, 20, 40, and 80, which reacted with antiserums (b, i, c) of homologous and two heterologous serogroups of leptospiras.

were reacted with a sulfuric acid-sulfhydryl reagent of Dische (BCyR)[13] and the colored reaction products measured in the ultraviolet region by means of a Beckman model DU spectrophotometer. The experiment, originally intended to confirm the identity of the pentose residue, discovered in addition, the presence of a second sugar component, a methylpentose. Figure 3 illustrates the color development of each of fraction 1 of *L. bataviae* and a reference solution of pure sugars of d(—)ribose and l(—)fucose (a methylpentose). The spectrums of fraction 1 and the reference solution of pure sugars, before the addition of cysteine, are represented in curves I. The primary colored reaction products, found fifteen to forty-five minutes after the addition of cysteine, are shown in curves II and are characteristic of pentoses. The secondary reactions products, found twenty-four hours after the addition of cysteine, are given in curves III and are characteristic of methylpentoses. The amounts of pentose and methylpentose in the samples were calculated from data obtained with d(—)ribose and l(—)rhamnose.

TABLE 1—Titration of Fraction 1 of Leptospira Canicola with Homologous Hyperimmune Serum

Antigen* diluted 1:	Homologous hyperimmune serum diluted 1: 15	30	60	120	240
1	4+	4+	4+	--	—
2.5	4+	4+	4+	1+	—
5	4+	4+	4+	3+	—
10	4+	4+	4+	4+	—
20	4+	4+	4+	4+	—
40	4+	4+	4+	4+	—
80	4+	4+	4+	3+	—
120	4+	4+	4+	±	—
160	4+	4+	4+	—	—
240	2+	1+	±	—	—

* Partially purified by precipitation with alkali in the presence of magnesium chloride (0.024 M) and concentrated to 0.5 per cent of the original culture volume.

Semiquantitative Complement-Fixation Titration.—In table 1 are summarized the results of a typical semiquantitative complement-fixation titration of a preparation of *L. canicola.* The preparation reacted with homologous hyperimmune serum obtained by immunizing rabbits with massive numbers of viable leptospiras. The product in this instance was unusually reactive and illustrated no anticomplementary activity although the fraction 1 concentrate was contained in 0.5 per cent of the original culture volume. Thus 0.25 ml. of the concentrate and the same volume of a 1:240 dilution of the antigen preparation reacted with diluted homologous hyperimmune serum. A zone of inhibition of complement fixation often was observed in the region of antigen excess. A representative semiquantitative assay of an aqueous cell-free extract (fraction 1) of *L. icterohemorrhagiae* with homologous and two heterologous hyperimmune serums is shown in table 2. Figure 4 is a graphic illustration of semiquantitative complement-fixation tests of preparations of fraction 1 of three serotypes of leptospiras with homologous and two heterologous hyperimmune rabbit serums. Arabic figures along the ordinate are numbers (that correspond to logarithms as illustrated in legend to fig. 4) of the reciprocals of the highest dilutions of hyperimmune serums (namely, the complement-fixation end points, as shown in table 2) at which a 2+ to 4+ reaction occurred with corresponding dilutions of antigen. The Arabic numerals along the abscissa represent decreasing amounts of antigen, namely, the aqueous preparation of fraction 1 diluted 1: 1, 2.5, 5, 10, 20, 40, and 80. The antigens employed were those of: (B) *L. bataviae,* (I) *L. icterohemorrhagiae,* and (C) *L. canicola.* The hyperimmune serums of the same serotypes are identified by small letters (b, i, and c). The relative specificity of each preparation and cross reactivity, particularly evident with *L. canicola* and *L. icterohemorrhagiae* antigens, is of the order commonly encountered with bacterial polysaccharides.[14] The cross reactions are of appreciable magnitude, but fraction 1 antigens react preferentially with homologous hyperimmune

TABLE 2—Complement-Fixation End Points of Fraction 1 of Leptospira Icterohemorrhagiae (Wijnberg)

Antigen diluted 1:	Hyperimmune rabbit serums* *L. icterohemorrhagiae*	*L. canicola*	*L. bataviae*
	COMPLEMENT-FIXATION END POINTS		
1	(Antigen anticomplementary at this density)		
2.5	480*	30	1**
5	480	60	1
10	480	120	1
20	960	120	1
40	960	30	1
80	240	15	1

* Reciprocals of highest dilution of hyperimmune serum reacting with corresponding amounts of the antigen.

** 2+ to 4+ complement-fixation reaction with undiluted hyperimmune serum but complete or nearly complete hemolysis in the next serum dilution tested (1:15).

serum at all density levels of the test antigen.

DISCUSSION

The chemical composition and complement-fixing properties of fraction 1 of leptospiras indicate that the cell-free extract contains a substantial amount of a major complement-fixing antigen system together with a complex pentosan polysaccharide material. The carbohydrate and a polynucleotide moiety appear to be closely associated in this extract, but there is no evidence that they are chemically bound. These facts together with the information that fraction 1 constitutes approximately 40 per cent of the dry weight of the leptospiral cell, indicate that the preparation is derived from the somatic part of the leptospiral organism. Analytical procedures have not sharply differentiated fraction 1 preparations of each of three serotypes of leptospiras. Measurements of absorption spectrums indicate the presence of similar or identical monosaccharide residues. The antigens, while reacting preferentially with homologous hyperimmune serums, illustrate serological group reactivity with heterologous hyperimmune serums. For the above reasons the preparations have been tentatively considered genus-specific materials, analogous in many respects to the somatic polysaccharide "C" substance of the pneumococcus.[15] The chemical composition and solubility properties further indicate that the complex polysaccharide of fraction 1 of leptospiras bears a certain resemblance to plant gums. The complex carbohydrates of plant gums are composed of hexose, uronic acid, pentose, and methylpentose residues.[16] Fraction 1 has been found to contain hexose, pentose, methylpentose, and glucosamine residues.[10,11] Analogous aqueous extracts from crude gum tragacanth, however, have no serological relationship to fraction 1 antigens, as determined by the complement-fixation reaction. The question of the apparent serotype-specificity of fraction 1 awaits further study.

Fraction 1 adsorbs readily on a naturally active, highly sorptive clay* material.[17] After separation by centrifugation, the aqueous phase is found to contain trace amounts of pentose and virtually no complement-fixation activity. Preparations have been precipitated from the aqueous phase, in the presence of magnesium chloride, by the addition of small amounts of alkali, and from acid solution at pH 2 by the addition of 2 per cent sodium tungstate solution. The precipitates contain 70 to 90 per cent of the starting pentose carbohydrate and virtually all of the complement-fixing principle in apparently undamaged state. Fraction 1 preparations have been treated with trypsin followed by dialysis. All of the active principle remained inside the dialysis sac. The above evidence together with the fact that the complement-fixing principle is heat stable is consistent with the conclusion that the complement-fixing moiety in fraction 1 is not protein. The active substance illustrates all the characteristics of a complex carbohydrate moiety.

The study of fraction 1 is incomplete. We have much to learn of its antigenic potency and more of its chemical characteristics. It obviously contains at least one major antigen system which should aid taxonomists in arriving at a more precise and rigorous method of classifying the leptospiras. When the new informations has been obtained and integrated, we shall be in a better position to judge what future practical applications these cell-free aqueous preparations of the leptospiras may have.

SUMMARY

Immunochemical studies of similarly prepared cell-free extracts of three serotypes of leptospiras are consistent with the conclusion that the complement-fixing principle is inseparable from a macromolecular pentosan-type polysaccharide material contained in fraction 1. Although fraction 1 preparations appear to be chemically very much alike, they are entirely different in the complement-fixation reactions.

Since it has not yet been possible by chemical means to separate the group reactive from the serotype reactive complement-fixing determinant, fraction 1 has been regarded as an inherently genus-specific cell-free extract of the leptospiral organism.

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* Attasorb, a product of Attapulgus Minerals and Chemicals Corp., Philadelphia, Pa.

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Infectivity and Pathogenicity of Vesicular Stomatitis Virus for Ferrets

T. KOWALCZYK, Vet. Dipl., D.V.M., M.S.; R. P. HANSON, Ph.D.; C. A. BRANDLY, D.V.M., M.S.

Madison, Wisconsin

OUTBREAKS of vesicular stomatitis among cattle and horses have occurred in the United States every few years. Where the virus survives, between epizootics, and the mechanism by which it spreads during each outbreak have been the subject of research in this laboratory.

The rapid spread of vesicular stomatitis, its frequent appearance in new foci on farms not adjoining those harboring active cases together with its prevalence during warmer seasons of the year, suggested the possibility of an arthropod vector. Experimental transmission by certain Diptera has been demonstrated by Ferris, *et al.*[4]

Reservoirs of the virus may exist inside or outside the epizootic area. Evidence has been advanced for enzootic foci in Mexico and in areas in the southern United States.[6] As yet there is no adequate evidence for or against the existence of latent infections among cattle or among small mammals and birds in the epizootic areas. A survey of the susceptibility of a number of species of animals to infection with vesicular stomatitis virus (VSV) revealed that the ferret may be readily infected by inoculation of the foot pad, by nasal instillation, and by cerebral inoculation.[7, 8] A severe and sometimes fatal disease ensued. This report concerns the recovery of VSV from infected ferrets after nasal instillation of the virus.

MATERIALS AND METHODS

Twelve, 1-year-old ferrets were obtained from the colony of the University of Wisconsin. The animals appeared to be in normal health, and body temperatures, recorded twice daily for a week prior to exposure to infection, were within the normal range. The animals were divided into two groups and exposed on successive days. Two ferrets were placed in each of a series of cages to facilitate collection of samples. Vesicular stomatitis virus of the New Jersey type, Wisconsin strain,[1] which had been maintained in embryos and had a chicken embryo $l.d._{50}$ titer of 10^{-4} per ml., was instilled into the external nares. Each ferret received 0.5 ml. of the inoculum. Beginning twelve hours after exposure, urine, saliva, feces, and blood were collected at four-hour intervals during the first two days, later three times daily. Blood was withdrawn by heart puncture from a different individual at each collection, whereas urine, feces, and saliva samples were obtained from all animals in each group. The fecal samples were suspended in 5 ml. of saline and centrifuged at 3,000 r.p.m. for thirty minutes. Two and one-half ml. of supernatant fluid were withdrawn, and an equal volume of a streptomycin and penicillin solution was added. Consequently, each half milliliter of inoculum contained 2,000 units of each antibiotic. After standing for thirty minutes, the material was inoculated into embryonating eggs. A composite of the six urine samples, collected at each interval, was treated with antibiotics as were the fecal samples. Saliva samples were collected from each animal by swabbing the oral mucosa with a sterile cotton swab. All swabs were placed together in a tube and shaken with 5 to 10 ml. of sterile saline solution for several minutes. After centrifugation, the supernatant fluid was treated with antibiotics as were the urine and fecal samples.

Ten, 9-day-old chicken embryos were inoculated in the allantoic chamber with 0.5 ml. of each material per egg. All eggs with embryos that had died within four days were chilled for six to twelve hours at about 6 C. after which the allantoic fluid was aseptically harvested and tested for sterility. If the sterility test failed to reveal bacterial growth, the allantoic fluid again was injected into ten eggs—five received the allantoic fluids alone and five received a mixture of the fluids admixed with specific VSV NJ-type serum of known titer (10^4 to 10^5 $l.d._{50}$ neutralized). The mixture of the fluid to be tested and the antiserum were incubated at room temperature for thirty minutes. Death of the first group of chicken embryos and survival of the second indicated recovery of the virus.

RESULTS

The first sign of infection of the ferrets was marked pyrexia which occurred between eighteen to twenty-four hours after exposure. Generally the temperature did not rise much over 104 F. but in a few cases it reached 107.2. The initial transient pyrexia was not associated with marked depression, lessened activity, decreased ap-

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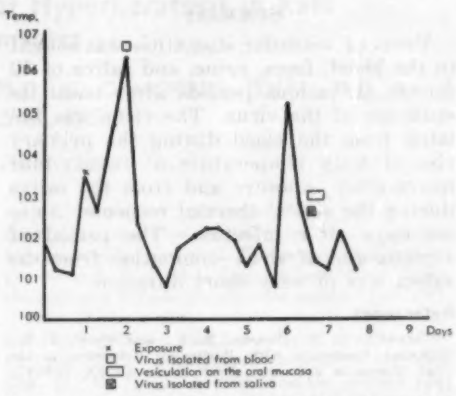
petite, or any other abnormality (graph 1).

A return of the body temperature to the normal range took place as rapidly as the rise, *i.e.*, in a matter of a few hours. During the following four days the ferrets were apparently normal and, finally, at the end of the fourth day, the body temperature showed a second rise which, however, did not reach the height of the first peak. The decline was associated with the appearance of vesiculation and ulceration of the mouth mucosa and foot pads. During the second elevation of temperature, there was impaired appetite, reluctance to move, and increased salivation among those animals with extensive mouth lesions. The temperature returned to normal quickly, and the healing of ulcerations proceeded relatively rapidly so that in five to seven days all the animals had recovered.

The attempts to recover the virus from feces and urine were not successful. Considerable difficulty was encountered in attempts to suppress the bacterial flora of these samples. The virus was isolated from the blood only at twenty-four hours after exposure and at the peak of the initial thermal response. Samples collected four hours before and four hours after this time did not show the presence of the agent. Of the saliva samples collected at four intervals only, those obtained during the decline of the second rise of temperature, when the vesicles had just developed, showed the presence of virus. All other blood as well as saliva obtained three times daily during the seven-day period were apparently free of the virus.



Graph 1—The isolation of vesicular stomatitis virus from a ferret in relation to the thermal response after intranasal exposure.

DISCUSSION

Even though the virus of vesicular stomatitis may perish readily outside the animal host, a knowledge of the time of its appearance in the saliva and in the blood is of significance toward an understanding of the epizootiology of the disease.

A viremia was observed by Shahan [10] in swine naturally affected with vesicular stomatitis; viremia occurred also among cattle and swine artificially exposed by Cotton,[3] Olitsky,[9] and Sorensen.[11] The ephemeral nature of the viremia and its characteristic low titer suggest that the blood of these animals can not be an important source of contamination or infection of blood sucking Diptera. The available evidence indicates that Diptera act as mechanical vectors and the virus-contaminated body surfaces are more important than is infective blood in transmitting vesicular stomatitis.

Cattle and horses, the common subjects of vesicular stomatitis epizootics, develop both viremia and hyperthermia within twenty-four hours after infection. This initial phase is the same as in the ferret but from this point the disease picture differs. Vesiculation and release of the virus into the saliva occurs immediately, or shortly, in adult cattle and horses. This phenomenon is delayed for several days or until the development of the second elevation of temperature in ferrets and certain other animals;[2] the virus may not be recoverable at all from still other species, e.g., man.[5] Infection in man has been observed in several laboratories, the disease usually being characterized by a biphasic temperature response unaccompanied by vesiculation. Calves, which on the basis of infrequent involvement during outbreaks were considered more resistant than adult cattle, have responded to inoculation with a biphasic temperature curve and successive vesiculation (unpublished data). Swine frequently developed double temperature peaks, vesiculation having been detected only during the second peak. Biphasic temperature response and longer incubation period suggest moderate resistance of the host to the virus.

SUMMARY

Virus of vesicular stomatitis was sought in the blood, feces, urine, and saliva of 12 ferrets at various periods after nasal instillation of the virus. The virus was isolated from the blood during the primary rise of body temperature at twenty-four hours after exposure and from the saliva during the second thermal response five to six days after infection. The period of viremia and of virus elimination from the saliva was of very short duration.

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Experimental Production of Hyperkeratosis in Rats and Hamsters

C. E. SCHOETTLE, M.S.; E. F. REBER, Ph.D.; C. C. MORRILL, D.V.M., Ph.D.; R. P. LINK, D.V.M., Ph.D.

Urbana, Illinois

HYPERKERATOSIS has caused significant losses in the cattle industry.

It was first observed in May, 1941, in New York State.[10] It is caused by one or more toxic materials which have occurred in a processed wheat concentrate,[8] alfalfa pellets,[9] timothy hay,[7] a German wood preservative,[11] a lubricant,[1] and a protein concentrate.[6] Recently, Bell [2] and Sikes [10] produced the disease by feeding penta-chloronaphthalene. Wagener [12] reported symptoms of hyperkeratosis in cats and dogs fed a hyperkeratogenic substance. Hansel *et al.*[4] determined the carotene and vitamin A content of plasma and liver of 24 calves and 1 cow used to study hyperkeratosis. Their data indicated that plasma vitamin A levels decreased rapidly after the hyperkeratosis-producing substance was fed but that feeding vitamin A in large amounts to cattle with hyperkeratosis resulted in increased plasma and liver vitamin A. However, when large doses of carotene were fed, plasma carotene increased, but plasma vitamin A did not, suggesting the possibility that the hyperkeratosis factor interfered with the conversion of carotene to vitamin A.

EXPERIMENTAL PROCEDURE AND RESULTS

Three experiments were carried out using hamsters and Sprague-Dawley strain of rats. Vitamin A analyses were made by the antimony trichloric method.[5] The animals were allotted to the different treatments so there was equal weight distribution and were weighed at weekly intervals. The rations contained either suspected or known hyperkeratogenic substances. The 33 per cent protein concentrate [6] contained 7 U.S.P. units of vitamin A per gram. A commercial chlorinated naphthalene product * was used in these studies.

Experiment 1.—The average initial weight of 10 male rats was 77 Gm. and of 10 females, 84 Gm. Five of each sex were fed the ration presented in table 1; the others were fed a commercial protein concentrate which would produce hyperkeratosis in cattle.[6] The rats were destroyed at the end of a 28-day period and their livers, kidneys, and thymus glands were weighed (table 2).

Four of the 5 males fed the protein concentrate developed priapism and 2 developed circular areas (1.0-1.5 cm. in diameter) of alopecia on the back (fig. 1). Of the 10 animals fed the protein concentrate, signs of pneumonia developed in 3 and noticeable discoloration of the liver in 5. Growth of all 10 rats was severely limited. Their liver weights were slightly increased, but their kidney weights did not appear to be influenced while their thymus glands averaged only one-third the weight of the thymus glands from the control group.

Histopathological examination revealed evidence of mild hyperkeratosis in the areas of alopecia in 2 animals (fig. 2). It also revealed that the liver discoloration was apparently due to a mild to moderate fatty

TABLE 1—The Composition of the Basal Ration,* Used in Experiment 1

Ration	Gm.
Vitamin-free casein	35.0
Lard	40.0
Lactose	10.25
Dextrose	10.25
Salt mixture (U.S.P. XIV)**	5.0
Cod liver oil †	0.5
Wheat germ oil (2 I.U., vitamin E/Gm.)	1.0

* The following amounts of vitamins were added per kilogram of ration: thiamine, 5.5 mg.; riboflavin, 9.0 mg.; nicotinic acid, 50.5 mg.; inositol, 134.0 mg.; choline, 1.3 Gm.; para-aminobenzoic acid, 25.0 mg.; folic acid, 0.65 mg.; biotin, 0.25 mg.; pyridoxine, 10.0 mg.; calcium pantothenate, 35.5 mg.; ascorbic acid, 650.0 mg.; vitamin B_{12}, 50.0 μg.; menadione, 1.45 mg.

** Composition:

Calcium carbonate		68.6
Calcium citrate		308.3
Calcium biphosphate		112.8
Magnesium carbonate		35.2
Magnesium sulfate		38.3
Potassium chloride		124.7
Dibasic potassium phosphate		218.8
Sodium chloride		77.1
Cupric sulfate	0.48 Gm.	16.2 Gm.
Ferric ammonium citrate	94.33 Gm.	
Manganese sulfate	1.24 Gm.	
Ammonium alum	0.57 Gm.	
Potassium iodide	0.25 Gm.	
Sodium fluoride	3.13 Gm.	
To make	100 Gm.	1,000 Gm.

† 1,000 U.S.P. units of vitamin A; 100 U.S.P. units of vitamin D/Gm. of oil.

From the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, and Agricultural Experiment Station, University of Illinois, Urbana.

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* A commercial chlorinated naphthalene product consisting of a mixture of tetra-, penta-, and hexachloronaphthalenes having a chlorine content of 62 per cent.

degeneration as evidenced by centrolobular vacuolation of hepatic cells (fig. 3). There was considerable evidence of nephrosis in the rats fed the protein concentrate mani-

Fig. 1—Rat showing area of mild depilation and hyperkeratosis.

fested by necrosis of tubules at the level of the medulla. The thymus of 2 animals fed the protein concentrate showed evidence of so-called accidental involution or sclerotic atrophy as manifested by a peripheral reticulosis (fig. 4).

Experiment 2.—Thirteen rats, weighing approximately 60 Gm., were fed the basal ration presented in table 3. The experiment was designed to evaluate the toxicity of chlorinated naphthalene, 400 mg. of which was mixed into each kilogram of ration. Portions of this were mixed with additional basal ration to make concentrations of 100 mg., 50 mg., and 25 mg. per kilogram of ration.

The effects of chlorinated naphthalene on livability, weight gains, liver weights, and liver vitamin A levels are presented in table 4. One rat fed 25 mg. of chlorinated naphthalene per kilogram of ration had areas of alopecia under the neck and between the legs. All 4 rats fed the ration containing 400 mg. of chlorinated naphthalene lost weight during the last half of the experiment, 1 had priapism, and 2 died before the end of the experiment. After eight weeks, the others were destroyed and the livers of all were removed, weighed, and analyzed for vitamin A. The chlorinated naphthalene appeared to have a depressing effect on liver vitamin A.

TABLE 3—The Composition of the Basal Ration Used in Experiment 2

Ration	Per cent
Crude casein	25.0
Wheat germ oil (2 I.U., vitamin E/Gm.)	3.0
Salt mixture (U. S. P. XIV)**	4.0
Lard	5.0
Cod liver oil †	2.0
Starch	29.8
Sucrose	30.0
Vitamin mix *	1.2

** *See* table 1.

* The following amounts of vitamins were added per kilogram of ration: thiamine, 8.0 mg.; riboflavin, 8.0 mg.; pyridoxine, 5.0 mg.; calcium pantothenate, 100.0 mg.; choline, 3.0 Gm.; nicotinic acid, 8.0 mg.; inositol, 1.0 Gm.; para-aminobenzoic acid, 0.3 Gm.; biotin, 250.0 μg.; and folic acid, 500.0 μg.

† 1,000 U.S.P. units of vitamin A; 100 U.S.P. units of vitamin D/Gm. of oil.

Experiment 3.—Forty-eight hamsters weighing approximately 50 Gm. each were divided into five

TABLE 2—The Effect of a Protein Concentrate on the Average Weight Gains and Organ Weights of Rats

	28-day weight gain (Gm.)	Organ weights, % body weight		
		Liver	Kidney	Thymus
MALES, CONTROL				
Ave.	171.0(5)*	5.0	1.2	0.3
Range	142.0-200.0	4.4-5.4	0.91-1.77	0.27-0.39
Standard deviation	20.0	0.4	0.4	0.02
Protein concentrate				
Ave.	29.0(5)*	6.8	1.2	0.1
Range	9.0-42.0	6.2-7.7	1.07-1.24	0.05-0.13
Standard deviation	15.0	0.6	0.1	0.03
FEMALES, CONTROL				
Ave.	66.0(5)*	4.5	0.9	0.3
Range	13.0-97.0	4.0-5.0	0.79-1.00	0.24-0.37
Standard deviation	29.0	0.4	0.1	0.06
Protein concentrate				
Ave.	5.0(4)*	8.3	1.2	0.1
Range	—1.0-7.0	7.9-9.1	1.16-1.29	0.08-0.14
Standard deviation	3.0	0.5	0.1	0.02

* Number of animals per treatment.

groups for the experiment. The five different rations fed for eight weeks consisted of the following: (1) commercial laboratory feed; (2) protein concentrate;* (3) basal synthetic ration; (4) basal synthetic ration plus chlorinated naphthalene; (5) basal synthetic ration minus vitamin A, plus carotene and chlorinated naphthalene. The compositions of these rations are shown in table 5.

The livers were removed, weighed, and vitamin A analyses made at the end of eight weeks or earlier when death intervened (table 6).

Both groups receiving chlorinated naphthalene showed considerable loss of weight during the experimental period. The hamsters fed the synthetic basal ration made an average total gain of 3.6 Gm. more than those fed the commercial laboratory feed. During the eight weeks of the experiment, no symptoms of hyperkeratosis were grossly visible in the hamsters fed the protein concentrate. Upon analysis of the livers, it was found that the hamsters fed the protein concentrate had an average vitamin A content of 4 U.S.P. units per gram of liver. The liver vitamin A was about twice as high in the hamsters fed the synthetic basal ration as in those fed the commercial laboratory feed. There was little difference in liver vitamin A of the two groups receiving the chlorinated naphthalene. The level of the chlorinated naphthalene in the two rations was probably higher than necessary to produce hyperkeratosis in eight weeks. Only 1 animal survived eight weeks when fed the rations containing chlorinated naphthalene, and it was in the group receiving vitamin A. The hamsters receiving vitamin A survived an average of twenty-eight days while those receiving carotene an average of nineteen days (table 6).

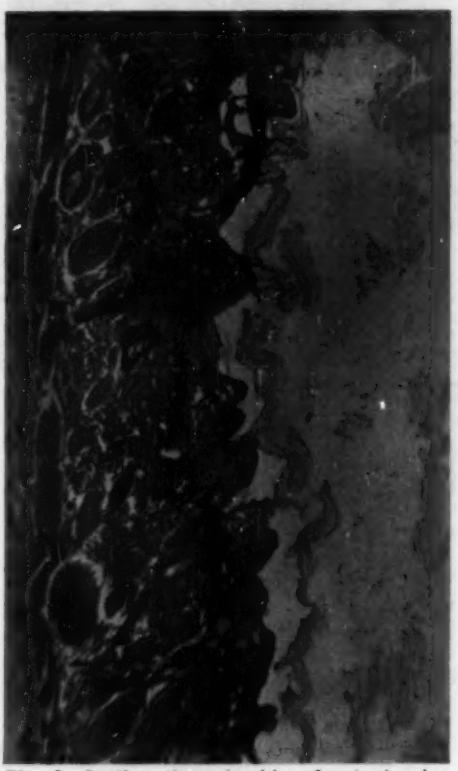

Fig. 2—Section through skin of rat showing mild depilation and hyperkeratosis (see fig. 1). Hematoxylin and eosin. x 50.

TABLE 4—The Effect of a Commercial Chloronaphthalene Product on Livability, and the Average Gains, Liver Weights, and Liver Vitamin A Content of Rats

Chloronaphthalenes, mg./kg. ration	Days alive on experiment	Total gains (Gm.)	Liver weights (Gm.)	U.S.P. units vit. A per liver
25				
Ave.	60(3)*	238.0	18.28	774
Range	58-63	178.0-275.0	14.10-21.95	562-1123
Standard deviation	2.2	43.0	3.22	249
50				
Ave.	60(3)*	222.0	19.84	806
Range	58-63	150.0-260.0	17.53-23.28	316-1386
Standard deviation	2.2	51.0	2.48	441
100				
Ave.	60(3)*	192.0	20.99	358
Range	58-63	96.0-257.0	14.43-29.10	82-624
Standard deviation	2.2	69.0	6.08	222
400				
Ave.	47(4)*	33.0	6.20	273
Range	41-55	5.0-62.0	4.95-8.28	88-416
Standard deviation	5.6	24.0	1.28	122

* Number of animals per treatment.

TABLE 5—The Composition of the Synthetic Rations Used in Experiment 3

	Ration		
	3 (%)	4 (%)	5 (%)
Crude casein	20.0	20.0	20.0
Cerelose	63.0	62.5	62.6
Lard	5.0	5.0	4.0
Cellulose	3.0	3.0	3.0
Salt mixture (U.S.P. XIV)**	4.0	4.0	4.0
Cod Liver oil †	1.5	1.5	....
Vitamin mix *	1.9	1.9	1.9
Wheat germ oil (2 I.U. vitamin E/Gm.)	1.5	1.5	1.5
Chloronaphthalene mixture	....	0.4	0.4
Carotene-crystalline (1.7 mg./Gm.)	....	....	1.0
Vitamin D (43b U.S.P. units of D/Gm.)	....	....	0.3

** *See* table 1.

* The following amounts of vitamins were added per kilogram of ration: thiamine, 8.0 mg.; riboflavin, 8.0 mg.; pyridoxine, 5.0 mg.; calcium pantothenate, 100.0 mg.; choline, 3.0 Gm.; nicotinic acid, 8.0 mg.; inositol, 1.0 Gm.; para-aminobenzoic acid, 0.3 Gm.; biotin, 250.0 μg.; folic acid, 500.0 μg.; and menadione, 140.0 mg.

† 1,000 U.S.P. units of vitamin A; 100 U.S.P. units of Vitamin D/Gm. of oil.

DISCUSSION

The principal effects of chlorinated naphthalene were on the thymus glands, liver, weight gains, and livability. When rats were fed a protein concentrate,[6] their liver weights were 7.5 per cent of body weight. Thymus glands were one third the size of those of the controls when compared on the basis of percentage of body weight.

Rats fed the ration containing 400 mg. of chloronaphthalene lived an average of forty-seven days. None of the other animals died during the experiment. Weight gains were about one ninth of those of the controls. The average total weight gains decreased about proportionally to the increase in chlorinated naphthalene in the ration. The 400-mg. level caused a decreased liver size as compared to the other levels. The livers of the control rats were 4.8 per cent of body weight. Animals on the 100- and 400-mg. levels had lower liver vitamin A content.

On the basis of weight gains in the hamsters, the protein concentrate was not as deleterious as were the rations containing chlorinated naphthalene, but the liver vitamin A content of those receiving the protein concentrate was considerably lower than that of the controls and lower than those receiving the rations containing chlorinated naphthalene. This may have been due to the low vitamin A or carotene content of the protein concentrate and to the effect of the hyperkeratogenic substance. The liver vitamin A was about twice as high in the hamsters fed the synthetic basal ration as in those fed the commercial laboratory feed. This difference was probably due to the difference in the carotene and/or vitamin A content of the feed.

Engel [3] reported that increasing the level of protein protected rats against fatty liv-

TABLE 6—The Effect of Hyperkeratogenic Feeds on Livability, and the Average Gains, Liver Weights, and Liver Vitamin A Content of Hamsters

Rations	Days alive on experiment	Total gains (Gm.)	Liver weights (Gm.)	U.S.P units vit. A per liver
COMMERCIAL LAB. FEED				
Ave.	59(7)*	33.3	5.12	365
Range	59-60	24.0-44.0	4.40-6.01	290-460
Standard deviation	0.38	6.7	0.57	55
PROTEIN CONCENTRATE				
Ave.	55(10)*	25.7	4.33	18
Range	54-58	16.0-37.0	3.50-5.13	5-49
Standard deviation	1.1	6.6	0.42	12
BASAL				
Ave.	55(7)*	36.9	5.35	789
Range	54-56	29.0-50.0	4.30-6.26	489-1,041
Standard deviation	1.0	7.2	0.54	176
BASAL PLUS CHLORINATED NAPHTHALENE				
Ave.	28(9)*	−20.3	2.74	52
Range	10-54	−11.0-−31.0	2.16-3.35	6-114
Standard deviation	15	5.3	0.43	38
BASAL MINUS VIT. A PLUS CAROTENE AND CHLORINATED NAPHTHALENE				
Ave.	19(8)*	−21.0	2.24	66
Range	10-43	−13.0-−32.0	1.88-2.62	25-133
Standard deviation	11	5.6	0.27	43

* Number of animals per treatment.

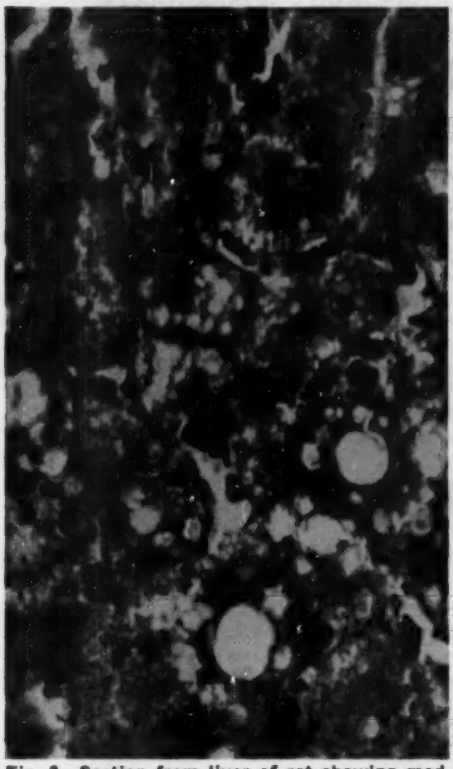

Fig. 3—Section from liver of rat showing moderate fatty changes as evidenced by vacuolation of hepatic cells. Hematoxylin and eosin. x 430.

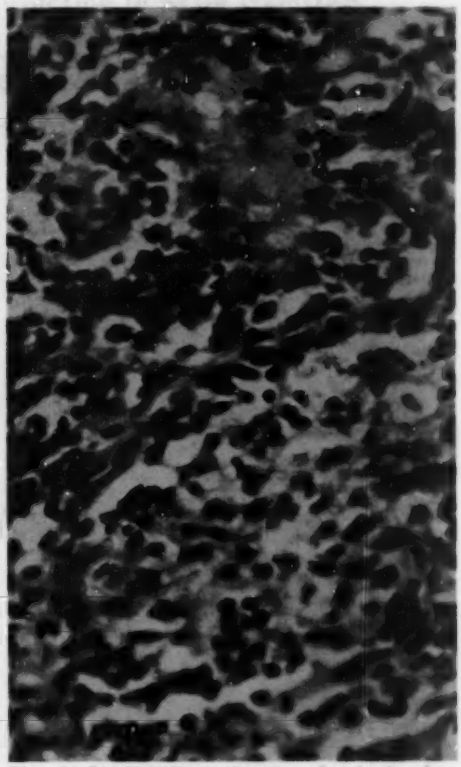

Fig. 4—Section from thymus of rat showing relative loss of thymocytes and prominence of reticulum. Hematoxylin and eosin. x 430.

ers and toxicity of hexachloronaphthalene included in the ration. The difference in weight gains of the hamsters fed the protein concentrate as compared to those fed the chloronaphthalene ration may be due, in part, to the higher protein content of the concentrate. However, the level of that protein did not appear to prevent low levels of vitamin A.

The rat, since it showed more symptoms than the hamster, appears to be the better laboratory animal for work on experimental hyperkeratosis.

SUMMARY

A protein concentrate which had produced hyperkeratosis in cattle, when fed to rats, limited their growth and shortened their lives. It produced alopecia with mild hyperkeratosis, mild to moderate fatty degeneration of the liver, low levels of vitamin A in the liver and small thymus glands in rats.

The chlorinated naphthalene produced alopecia, low levels of vitamin A in liver, limited growth and shortened life of rats. Hamsters fed chlorinated naphthalene and vitamin A lived longer than those fed chlorinated naphthalene and carotene.

The rat appeared to be more susceptible to the toxic action of a hyperkeratogenic agent than the hamster.

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Experimental *Vibrio Fetus* Infection in Male Hamsters

M. RISTIC, Dipl. Vet., D.V.M., M.S.; D. A. SANDERS, D.V.M.;
F. YOUNG, M.Ag.

Gainesville, Florida

EVIDENCE that the male hamster may be infected experimentally with *Vibrio fetus* and that such an animal transmits infection by coitus to uninfected females was presented in a previous paper.[4] The report, however, was limited to the study of a single Vibrio strain of ovine origin. Previous studies showed that variations in virulence of *V. fetus* strains may be associated with differences in their antigenic structure.[5] The wide range of the degree of serological relationship among Vibrio strains and the limited knowledge regarding their specific antigenicity [1,3,6] make it of interest to extend investigations to a greater number of strains.

This study concerns the pathogenicity of 18 *V. fetus* strains of bovine, ovine, porcine, and human origin for male hamsters. Antigenic relationship of the human strains to some of those of bovine and ovine origin was established by cross reaction as determined by reciprocal agglutination test, conducted with Vibrio antigens and specific antiserums produced in rabbits.

In addition, preliminary observations were made on the hypersensitivity reaction of infected hamsters to whole *V. fetus* cell antigens, administered intradermally.

MATERIALS AND METHODS

All hamsters used in these experiments were young adult males approximately 80 to 100 Gm. in weight.

Seven of the nine bovine Vibrio strains investigated were isolated from the stomach content of aborted fetuses, while two were recovered by the vaginal tampon method from apparently normal animals. The five ovine strains were associated with abortion, while the porcine strain was recovered from the blood of an animal having a history of a gangrenous condition. The three human strains originated from patients in which a subacute bacterial endocarditis was diagnosed.

The organisms were cultivated in 16-oz. prescription bottles of Albimi Brucella agar which were slanted to provide the greatest possible surface area. Since six Vibrio strains could not be adapted to grow on solid mediums, these organisms were produced in Albimi Brucella broth. In order to increase the solidity of the agar and the viscosity of the broth, 5.0 Gr. and 1.5 Gr. of plain agar were added to each liter of the medium, respectively.

Incubation was at 37 C. in an atmosphere of approximately 10 per cent carbon dioxide for seventy-two to ninety-six hours. In preparing the inoculum, the resultant growth was washed from the agar surface with sterile 0.85 per cent sodium chloride solution, or a characteristic layer of subsurface growth was collected from Albimi Brucella broth. The suspension was filtered through sterile glass wool, washed with sterile saline solution, and standardized so that 0.2 ml. of this suspension added to 5.0 ml. of saline gave a reading of 55 per cent light transmission in the Leitz photoelectric colorimeter with a 550 μ green filter. The density approximated tube 3 of the McFarland nephelometer.

Experiment 1, Study of Pathogenicity of Vibrio Fetus.—Eight experimental hamsters per strain of *V. fetus* received intraperitoneally 0.5-ml. suspension of the organisms on days 1 and 3. Two control animals were inoculated in the same manner with saline solution used for washing of each strain.

The infected animals were observed for four weeks. Two of the experimental animals per strain studied were destroyed weekly, following the last Vibrio inoculation, while the controls were destroyed at the termination of the experiment.

The testes were removed and each was cut in three to five pieces under aseptic conditions. The individual pieces were placed in tubes of Albimi Brucella broth. The incubation procedure was similar to that used for preparation of the inoculum. The identification of *V. fetus* was attempted up to seven days of incubation utilizing gram-staining technique and the agglutination test,[2] employing high titer rabbit antiserums.

Experiment 2, Study of Hypersensitivity Reaction.—The dermal hypersensitivity to whole *V. fetus* cell antigen was determined by injecting hamsters as described above. Four animals were inoculated with each of five selected Vibrio strains found to be pathogenic for hamsters. A like number of controls were confined in separate boxes.

Fifteen days following the second inoculation, all hamsters were given two intradermal injections, 1 cm. apart, of 0.01 ml. of the formalin- and the heat-killed antigens, in the posterior sternal

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region. The formalin-killed antigen was prepared by suspending the cells in 0.3 per cent formaldehyde-saline solution and storing in the refrigerator. After forty-eight hours, the cells were washed three times with sterile saline to remove excess formalin. The final product was prepared by suspending the packed cells in an equal volume of sterile saline. The heat-killed antigen was prepared by suspending the cells in saline, heating in a water bath at 56 C. for two hours, washing three times, and finally suspending as described for formalin-killed antigen.

All tests were read and interpreted within twenty-four to forty-eight hours after the intradermal injections. Presence of hyperemic edema of firm consistency, measuring 0.40 cm. or more in diameter, was arbitrarily considered to be a positive reaction. Three days following the injection, all animals were killed and the testes were cultured as described in experiment 1. Blood serums agglutinin titers were determined by the agglutination test.[2]

RESULTS

Experiment 1.—A summary of data pertaining to persistence of *V. fetus* in the testes of experimental animals is presented in table 1. Four of the nine bovine strains were recovered in pure culture from one or both testes of hamsters infected with *V. fetus* during a period up to three weeks following the last inoculation. The highest incidence of infectivity was established by strain 169. There was no good correlation between the gross lesions of the testes and bacteriological findings.

All ovine strains except 543 proved pathogenic for male hamsters. Strains 2221 and 4849 were recovered during the first week following inoculation while the others persisted through the four-week period.

The three human strains produced infection. Strain 1134 possessed a higher pathogenic potentiality in regard to incidence of infection and the degree of gross pathological change of the testes. Infection of the testes established by this strain was maintained in one animal throughout the period of observation. Data concerning serological cross reaction of the human strains with antiserums of four *V. fetus* strains of animal origin are presented in table 3.

Strain 8446 of porcine origin failed to produce infection in any of the animals inoculated and did not exhibit cross reaction with any of 16 specific antiserums.

Some of the strains studied induced macroscopic organic change other than those observed in the testes. These consisted of fibrin deposits on the splanchnic organs and liver-diaphragm adhesions. Attempts to culture Vibrio from the hepatic and splenic tissues of such animals failed. The gross lesions of *V. fetus*-infected testes are shown in figure 1 and 2. Microscopic changes occurring in the hamster testes resulting from intraperitoneal inoculation have been reported in a previous study.[4]

TABLE 1—Pathogenicity of Vibrio Fetus for Male Hamsters Following Intraperitoneal Inoculation

Vibrio strain	Origin	No. exptl. anim.	Autopsy time in weeks after inoculation								Totals	
			1		2		3		4			
			A	B	A	B	A	B	A	B	A	B
	Bovine											
U.F.	Aborted case	8	1	2	0	1	0	1	0	0	1	4
169*	Aborted	8	2	1	1	1	1	1	0	1	4	4
P_1	Aborted	8	0	0	0	0	0	0	0	0	0	0
153*	Aborted	8	1	2	1**	1	0	1	0	0	2	4
RL51	Aborted	8	0	1	0	0	1**	1	0	0	1	2
426*	Aborted	8	1**	2	1	0	0	1	0	0	2	3
R3	Aborted	8	0	1	0	0	0	0	0	0	0	1
DL Fash	Nonaborted case	8	0	0	0	0	0	0	0	0	0	0
19*	Nonaborted	8	0	0	0	0	0	0	0	0	0	0
53D178	Ovine	8	1	2	1	1	1	1	1	1	4	5
K	Ovine	8	1	1	1	2	1**	1	1	0	4	4
543	Ovine	8	0	0	0	0	0	0	0	0	0	0
4849*	Ovine	8	1	1	0	1	0	1	0	0	1	3
2221	Ovine	8	1	1	0	0	0	0	0	0	1	1
1134	Human	8	2	2	1	2	1	1	1	1	5	6
1308	Human	8	1	1	1	1	0	1	0	1	2	4
1154	Human	8	1**	0	1	0	0	0	0	0	2	0
8446*	Porcine	8	0	0	0	0	0	0	0	0	0	0

A—Number of animals infected; B—Number of animals showing one or two atrophied testes.

* Inoculum prepared in Albimi Brucella broth; ** *Vibrio fetus* identified by gram-staining technique only.

Control animals for each of the above strains were bacteriologically negative and no visible organic change was observed at termination of the experiment.

TABLE 2—Dermal Hypersensitivity Reaction of Infected Male Hamsters to Vibrio Fetus Antigens

Vibrio fetus strains	Experimental animals antigen				Control animals antigen			
	Formalin killed		Heat killed		Formalin killed		Heat killed	
	+	−	+	−	+	−	+	−
U.F.	(1)4	0	4	0	1	3	3	1
53D178	(1)3	1	4	0	1	3	3	1
K	(3)4	0	4	0	0	4	4	0
1308	(1)1	3(3)	2	2	1	3	3	1
1154	4	0	4	0	0	4	3	1
1134	(2)3	1(1)	4	0	1	3	4	0

Number of animals: + positive skin reaction; − negative skin reaction.
( ) Number of animals with infected testes.

All control animals were bacteriologically negative and no gross pathological changes were observed.

Experiment 2.—In general, the experimental animals manifested varying degrees of dermal reaction to both formalin- and heat-killed antigens. The reaction to each antigen was apparent within ten hours. It became maximal during twenty-four to forty-eight hours following the injection after which it slowly receded. Reaction to formalin-killed antigens was occasionally accompanied by a varying degree of surrounding erythema 5 to 8 mm. in diameter, with slight central blanching or necrosis.

Four of the 24 control animals exhibited a positive dermal reaction to formalin-killed antigens. The differences between the reaction to heat-killed antigens observed in control and experimental animals were not significant.

The correlation between testes infection of experimental animals and their dermal hypersensitivity reaction was established in some but not all animals. Blood serum of all *V. fetus*-inoculated animals contained agglutinins for the respective strains at titers of 1:160 to 1:1,280. All controls were serologically and bacteriologically negative. The results of this experiment are summarized in table 2.

DISCUSSION

An important finding of experiment 1 was the gonadotropism manifested by all pathogenic *V. fetus* strains. Such a tissue predilection further demonstrates the ability of the organisms injected into the peritoneal cavity to transgress certain host barriers before invading the testes. While the question regarding persistence and location of *V. fetus* infection in bulls is unknown, the tissue affinity of this organism, without reference to other aspects of host-parasite interaction, suggests ascending testes infection.

Under the conditions of this experiment, Vibrio strains displayed variation in their pathogenic potentialities. Of the strains employed in this study, the organisms of bovine origin were less virulent than the strains from ovine and human sources. A number of cultures did not demonstrate any pathogenic character for hamsters.

The differences in pathogenicity of organisms studied may be attributable to inherent differences among strains, since most of these, with exception of the por-

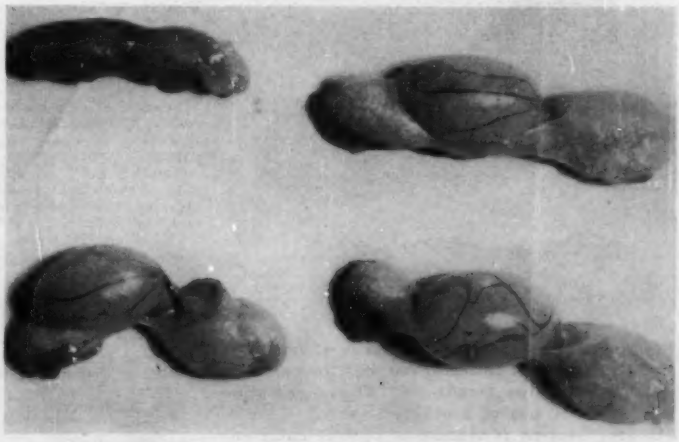

Fig. 1—(Left) Marked and slight atrophy of testes of hamsters at three weeks following intraperitoneal inoculation with Vibrio fetus strain 1134. (Right) Testes of control hamster.

TABLE 3—Cross-Agglutination Reaction Between Vibrio Fetus Strains of Human and Animal Origin

Human *V. fetus* antigens	*Vibrio fetus* antiserums*					
	**Bovine		**Ovine		**Human	
	RL51	R3	K	543	1154	1134
1134	P320	—	P80	—	80	1280
1154	—	1280	160	640	P1280	160
1308	P160	—	80	80	160	P160

* All antiserums were inactivated by heating at 56 C. for thirty minutes; ** origin of strains.
P Incomplete or partial agglutination of the antigen at the given dilution; — no demonstrable agglutination.

cine strain, have been shown to possess an antigenic fraction serologically shared to a certain extent with other strains.

An interesting similarity of two ovine strains, 53D178 (Colorado) and K (Wisconsin) was demonstrated by their ability to persist and produce changes of the testes, as well as by the degree of their serological cross reaction.

Comparison of the cultures of human origin indicates that although all three were capable of establishing an infection, strain 1134 had the greater tendency to persist and produce more drastic changes of the testes. Vinzent [7] reported three cases of human *V. fetus* infection in France, resulting in abortion in one instance. The author was able to agglutinate cells of 2 Vibrio strains of animal origin using serums of his patients. Results of our investigations show a cross reaction of three human strains from this country as determined by agglutination tests conducted with two ovine and two bovine antiserums. Whether *V. fetus* strains are truly bovine, ovine, or human or whether such a designation frequently means only priority in isolation is a subject of further research.

Fig. 2—(Lower) Section of testicle of hamster two weeks following intraperitoneal inoculation with Vibrio fetus strain 169. The organ contains pus of a caseous nature. (Upper) Section of testicle of control hamster.

Differences in response of sensitized and control animals to the two types of whole cell antigens administered intradermally could depend upon various factors. The Vibrio strains employed, methods of preparing the antigens, and individual differences in susceptibility and reactivity of animals may influence results. It is possible that sensitization of the animals infected was directed against allergenic factor or factors located within the whole Vibrio cell. In such cases, the compact flagellar-somatic unit of formalin-fixed *V. fetus* cells would induce more specific reaction than the heat disintegrated antigen. Results of this study indicate feasibility of further investigation of the dermal hypersensitivity reaction.

SUMMARY

Vibrio fetus strains of bovine, ovine, and human origin produced infection of the testes in male hamsters following intraperitoneal inoculation. Eleven of the Vibrio strains were recovered from the testes in which gross pathological lesions were observed. *Vibrio fetus* strains of human origin were serologically related to some of those of animal origin. No cross reaction was observed between the porcine strain and any of the others studied. A degree of dermal hypersensitivity was demonstrated with formalin-killed antigens, while heat-killed antigens produced essentially equal reactions in both infected and control animals.

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